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INDOLE DERIVATIVES IN CONNECTION WITH A DIET DEFICIENT IN TRYPTOPHANE. II.

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The well established fact that tryptophane is necessary both for body maintenance and growth gives rise to considerable interest as to what metabolic paths this amino acid travels in the animal organism. From the various studies thus far reported, it appears that kynurenic acid is the only *definitely characterized* intermediary substance that has been related beyond doubt to the physiology of tryptophane, and kynurenic acid is generally regarded as a "shunt" product. In view of the undoubted importance of tryptophane in contrast to the rather meager knowledge available concerning the chemical alterations which it undergoes in the body, Dakin (1922, p. 97) has stated: "Further investigation of the normal metabolism of tryptophan is highly desirable." What compounds in addition to kynurenic acid play a rôle therein?

A method of approach which has appeared attractive to the writer has been that of administering possible metabolic intermediates or likely progenitors of such intermediates to animals deprived of a particular indispensable unit. Either the nitrogen balance or the change in body weight may be used as an index of the availability of a substance thus tested. Studies based upon the nitrogen balance method were undertaken by Abderhalden in 1915. The following year Asayama working in Hopkins' laboratory demonstrated that the animal's synthesis of kynurenic acid from tryptophane is not a reversible one. Rats in a condition of stress for tryptophane did not grow when given kynurenic acid. Since then, several further similar studies of tryptophane, lysine, histidine, and cystine have been published. In an earlier paper

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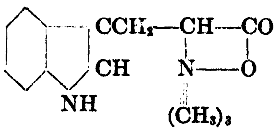
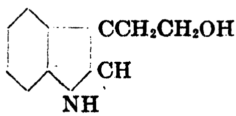
on tryptophane (1927), the writer briefly reviewed some of these investigations. Most notable were the observations of Cox and Rose (1926) and Harrow and Sherwin (1926) that *dl*-β-4-imidazole lactic acid could to a large extent replace histidine in the diet. The corresponding pyruvic acid was likewise found physiologically available, though to a lesser extent.

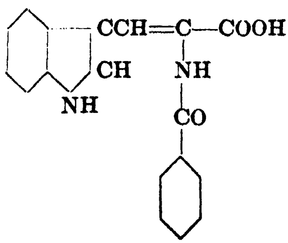
In view of these significant findings in regard to histidine, it became important to investigate in a like manner various tryptophane derivatives. 3-Indole aldehyde and *l*-β-3-indole lactic acid were found without effect when fed to rats upon a diet deficient in tryptophane (Jackson, 1927). In the present paper[1] is recorded an investigation of a series of nine additional indole derivatives with position 3 side chains as shown in the accompanying formulas.



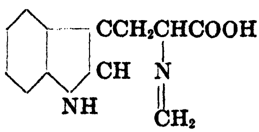
The betaine of tryptophane



Indole ethyl alcohol

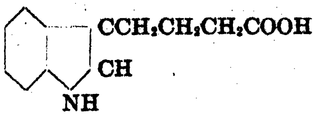


β-Indole α-benzoylamino-acrylic acid

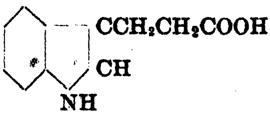


The formaldehyde-tryptophane product (Homer's formula)

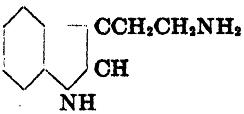
[1] A preliminary report was made at the meeting of the American Society of Biological Chemists at Ann Arbor, 1928.



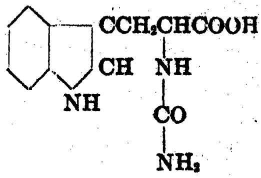
Indole butyric acid



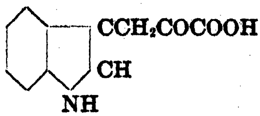
Indole propionic acid



Indole ethylamine



β-Indole α-uraminopropionic acid



Indole pyruvic acid

Of the materials enumerated, indole propionic acid, indole butyric acid, indole ethylamine, and indole ethyl alcohol may not at first appear to be particularly promising for the purpose of this study, but it must be kept in mind that all of these may at least undergo oxidation and thereby be transformed to intermediates which themselves might be suitable for tryptophane synthesis. Thus indole butyric acid by β-oxidation would be converted to indole acetic acid perhaps via indole acetaldehyde or some other equally interesting intermediate. Much the same argument holds for indole ethylamine and indole ethyl alcohol which have been shown respectively by Ewins and Laidlaw (1913) and Ward (1923) to be oxidized to indole acetic acid in the animal body. Sullivan (1922) has detected indole ethylamine in the urine of pellagrins.

Indole pyruvic acid, in view of the generally emphasized close physiological relation between α-amino acids and the corresponding pyruvic acids, appeared to be quite deserving of investigation. Moreover, Ellinger and Matsuoka (1920) have discovered that it gives rise to an increased kynurenic acid output. It possesses,

therefore, a very distinctive metabolic property in common with tryptophane. β-Indole α-benzoylaminoacrylic acid is interesting from the physiological standpoint of the dehydrogenation theory of amino acid oxidation (*cf.* Wieland and Bergel, 1924, and Dakin, 1926). Further the structural similarity of the substance to hippuric acid, which is so closely related to the corresponding amino acid glycine, is to be noted. The betaines of the amino acids found in proteins are of considerable importance—an importance accentuated by the recent discovery of ergothioneine in mammalian blood. Hypaphorine, the betaine of tryptophane, occurs in nature in the plant kingdom (*cf.* van Romburgh and Barger, 1911).

β-Indole α-uraminopropionic acid is significant since in it the amino group of tryptophane is replaced with another of common physiological importance—the urea group. Fearon and Montgomery (1924) have discussed the possible substitution of the α-amino group as a step previous to deamination (*cf.* Lippich, 1908; Dakin, 1926). The uramino derivative of tryptophane is of interest in this connection. Fearon and Montgomery themselves have in particular suggested the possibility of the chemical alteration of the $-NH_2$ to the $-N{=}CH_2$ grouping in the deamination process. The corresponding $-N{=}CH_2$ derivative of tryptophane, therefore, was also taken under consideration.

EXPERIMENTAL.

The general experimental technique described in the earlier publication was again employed with the following modifications. Owing to reports that intestinal bacteria possess the power of synthesizing tryptophane, it was thought best to confine the rats in cages equipped with false bottoms. All animals numbered 52 or beyond were thus prevented access to the feces. The lard in the diet was increased from 20 to 25 per cent, the extra 5 per cent displacing the 5 per cent of cod liver oil, which material was now given separately, 5 drops (100 mg.) per each rat per day. Vitamin B (what has hitherto been called vitamin B) was administered also separately in the form of 150 mg. of yeast concentrate[2] mixed with an equal amount of dextrin. 25 mg. of tryptophane were

[2] Lots 1030 and 1042 of Yeast Vitamine Harris were made into a mixture which was tested for use in these experiments.

added to each 100 gm. of the basal diet in order to bring about body maintenance as nearly as possible. The positive effect of any derivative might then be more accurately observed in departures of the growth curve from the horizontal line. In turn, the amount of tryptophane in the control diet was lowered from 300 to 250 mg. per 100 gm. and the derivative to be tested was incorporated in the diet in amounts equivalent to 250 mg. of tryptophane; *i.e.*, 1 or 2 equivalents, etc.

Preparation of the Indole Compounds.

The betaine of tryptophane was prepared according to van Romburgh and Barger (1911). 6.0 gm. of pure tryptophane upon methylation gave 6.5 gm. of crystalline methyl ester of the quarternary iodide of excellent quality (4.5 gm. of the nitrate were recovered from the mother liquor) which was hydrolyzed and converted to the nitrate. This in turn was treated with sodium carbonate solution to give the crude free base (yield 4.4 gm.). Recrystallization from 95 per cent alcohol and ether gave 3.7 gm. of beautiful white crystals of the dihydrate. The following data are for the anhydrous substance. M. p., 249° (corrected); m. p. (van Romburgh and Barger), about 255°. Total nitrogen,[3] per cent: found, 11.05; theoretical, 11.38.

The β-indole α-benzoylaminoacrylic acid was prepared according to Ellinger and Flamand (1908). From 3.0 gm of 3-indole aldehyde were secured about 4 gm. of the crude product which upon four fractional crystallizations from 70 per cent alcohol with simultaneous bone-black treatments yielded 1.7 gm. of white crystals. M. p., 232–234° (corrected); m. p. (Ellinger and Flamand), 232–234°. Total nitrogen, per cent: found, 9.01; theoretical, 9.15.

The indole butyric acid[4] was synthesized by applying the Fischer indole synthesis to the phenylhydrazone of ethyl hydrogen α-ketopimelate. The acid crystallized from a benzene-petroleum ether mixture as glistening rhombic plates. M. p., 123–124° (corrected). Total nitrogen, per cent: found, 6.77; theoretical, 6.90.

The indole propionic acid[5] was prepared according to Kalb, Schweizer, and Schimpf (1926). The material after a final crystallization from hot water consisted of white needles. M. p., 133–134° (corrected); m. p. (Kalb, Schweizer, and Schimpf), 134°. Total nitrogen, per cent: found, 7.32; theoretical, 7.41.

The indole ethylamine was prepared according to Majima and Hoshino (1925). From 15.6 gm. of indole were secured 13 gm. of indole acetonitrile which upon reduction yielded 7 gm. of the crude base. This was finally

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[3] All nitrogen determinations were made by the Kjeldahl method.

[4] A complete acount of the synthesis will be published shortly in collaboration with R. H. F. Manske.

[5] This material was kindly supplied by Dr. Manske.

purified by adding an ether solution of hydrochloric acid to the material dissolved in a mixture of 400 cc. of ether and 40 cc. methyl alcohol. The material was once more crystallized from alcohol and ether. The final yield of the hydrochloride was 3.6 gm. The crystals were white with just a very slight tinge of lemon-yellow. M. p., 251–253° (corrected); m.p. (Majima and Hoshino), 248–249°. Total nitrogen, per cent: found, 14.04; theoretical, 14.25.

The indole ethyl alcohol was prepared according to the elegant biological method of Felix Ehrlich (1912). 10 gm. of tryptophane gave 7.2 gm. of nicely crystalline crude tryptophol.[6] (The theoretical yield here would be 7.9 gm.) This was recrystallized from 25 per cent alcohol to give 3.7 gm. of first crystals and 2.6 gm. of additional very slightly colored material. The first fraction consisted of beautiful colorless plates. M. p., 57–59° (corrected); m. p. (Ehrlich), 59°. Total nitrogen, per cent: found, 8.62; theoretical, 8.69.

The formaldehyde condensation product of tryptophane was prepared according to Homer (1913). 2.0 gm. of pure tryptophane yielded 2.2 gm. of the derivative. The substance was slightly yellow, as described by Homer. M. p., 230–240° (corrected); m. p. (Homer), 225–240° and 235–240°. Total nitrogen, per cent: found, 10.75; theoretical, 11.11. It seems somewhat doubtful that this substance contains the $-N{=}CH_2$ group as suggested by Homer. Her work shows that the second molecule of water of crystallization apparently cannot be removed under the temperature which brings about decomposition of the compound. Furthermore, Franzen and Fellmer (1917) have shown that this extra molecule of "water of crystallization" is characteristic of the formaldehyde condensation products of other amino acids and also of various salts of these products. They, therefore, have suggested the $-NH-CH_2-OH$ group instead of $-N{=}CH_2$.

The β-indole α-uraminopropionic acid was not found described in the literature. It was prepared in general according to the directions of Lippich (1908) for the production of corresponding derivatives from other amino acids. 5 gm. of pure tryptophane were refluxed for 5 hours with 12.8 gm. of urea and 320 cc. of saturated barium hydroxide. The barium was removed with carbon dioxide and the filtrate was evaporated *in vacuo* to a volume of 75 cc. Acidification with acetic acid gave 4.7 gm. of crude material. Three crystallizations out of 50 per cent alcohol gave 3.30 gm. of crystalline substance (prisms and plates). M. p., 200° (corrected). Total nitrogen, per cent: found, 16.80; theoretical, 17.00. It may be noted that this material was pure white until the second crystallization. It then assumed a very slight purplish cast both in the crystals and in the mother liquor; the color still remained after the final crystallization. This phenomenon is suggestive of the pinkish cast which even pure 3-indole acetic acid may assume. The identity of the uramino derivative of tryptophane

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[6] The writer wishes to thank Dr. Frey of the Fleischmann Laboratories, New York, for a generous supply of starch-free yeast.

was further established by converting a small amount of it to the hydantoin which has previously been described by Majima and Kotake (1922).

The indole pyruvic acid was prepared according to Gränacher, Gerö, and Schelling (1924). 3-Indole aldehyde was condensd with rhodanine. The resulting substituted rhodanine was hydrolyzed to indole sulfhydryl-acrylic acid from which the sulfur was eliminated to give the desired indole pyruvic acid. Preparation 1: The crude pyruvic acid prepared from 3 gm. of the indole aldehyde was twice precipitated from boiling acetic acid to give 0.6 gm. of brown micro crystals (containing 1 mol of acetic acid of crystallization). Preparation 2: The crude pyruvic acid prepared from 5 gm. of the indole aldehyde was twice fractionally precipitated from ether with petroleum ether to give 1.8 gm. of a very light brown material. Total nitrogen, per cent: found, 7.27; theoretical, 6.90. Examination of Preparation 2 for amino nitrogen in the Van Syke apparatus showed that the "amino nitrogen" was 3 per cent of the total nitrogen. The melting points of both preparations described here were indefinite; it seems that a *fairly sharp* melting point for indole pyruvic acid has not been reported.

The tryptophane used both in the construction of diets and in the synthesis of the derivatives was prepared in quantity essentially according to Onslow's (1921) modification of the original method of Hopkins and Cole (1901). The preparation was beautifully crystalline. Total nitrogen, per cent: found, 13.60; theoretical, 13.72. A polarimetric examination revealed it to be the levo form.

Diets.

The diets employed are indicated in Table I. Generally the rats were placed upon Diet 210 until they reached 115 to 120 gm. in body weight, and then were transferred to the tryptophane-deficient diet, No. 211, for a period of about 44 days. Most of the animals eventually exhibited a remarkably constant body weight maintenance. Next, the derivative to be studied along with sufficient sodium bicarbonate to neutralize any free acid present was introduced in 1 or 2 equivalents (equivalents of 250 mg. of tryptophane per 100 gm. of food; see Table I) most often for 24 days. The deficient diet was then restored for a period, and finally tryptophane was incorporated in the diet to demonstrate that the animal still possessed capacity for growth. Thus each experiment was controlled within itself. In addition, separate animals were always kept on both the deficient diet and the deficient diet plus tryptophane to show simultaneously that no abnormal environmental factors were operating.

TABLE I.

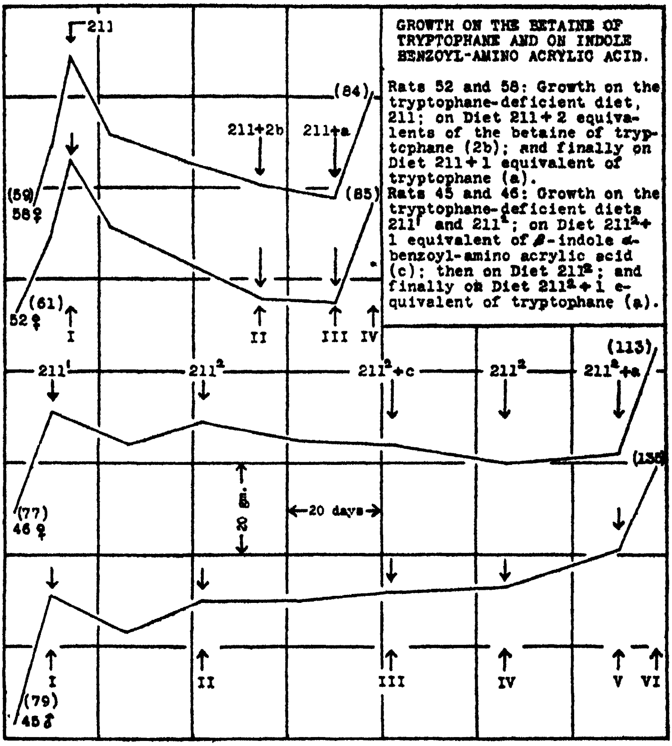
Composition of Diets.

	Diet No.			Diet 211 supplemented with derivatives.		
	210	211	E	Diet No.	Compound.	Amount of compound added per 100 gm. Diet 211.
	gm.	*gm.*	*gm.*			*mg.*
Casein, technical.	18.0			211 + a†	Tryptophane.	250
Casein digest (digested				211 + b	Betaine of tryptophane.	302
in acid).		14.4		211 + c	β-Indole α-benzoylaminoacrylic acid.	375
Cystine.		0.3		211 + d	Indole butyric acid.	249
Tyrosine.		0.3		211 + e	Indole propionic acid.	232
Tryptophane.		0.025		211 + f	Indole ethylamine hydrochloride.	241
Dextrin, white.	36.0	39.0	50.0	211 + g	Indole ethyl alcohol.	197
Sucrose.	15.0	15.0	25.0	211 + h	Formaldehyde-tryptophane product.	309
Salt mixture.*	4.0	4.0		211 + i	β-Indole α-uraminopropionic acid.	303
Agar (ground).	2.0	2.0		211 + j	Indole pyruvic acid.	249
Lard.	25.0	25.0	25.0			(323)‡
Total.	100.0	100.0	100.0			

* The salt mixture used was prepared according to Osborne and Mendel (1919).

† Diets containing the indole derivatives are designated by appending letters to 211. The single letter stands for one equivalent of the derivative; when other than one equivalent is employed, the proper coefficient is inserted.

‡ In the case of Preparation 1 (j), 323 mg were used in order to allow for the 1 molecule of acetic acid of crystallization.



Charts I to VII. The initial portions of the growth curves represent growth upon the unhydrolyzed-casein diet, Diet 210 (no legend shown). The downward arrows indicate dietary changes. The first number above a downward arrow denotes the diet introduced. Upward arrows indicate points and intervals for the purpose of describing the sequence of the experiments and for the purpose of recording food consumption (Tables II and III). In Tables II and III, Period I is employed always to designate 12 days on Diet 210, ending at the point marked I in the charts. Either three or four animals were used in connection with the testing of any one derivative, although the growth curves for only one or two are presented.

Chart I. Diet 211, which was fed in an experiment upon a group of animals including Rats 52 and 58, was prepared from a casein digest to which the cystine, tyrosine, and tryptophane were added in the wet way. There were indications that the subsequent drying down process caused the tryptophane to decompose. Diets 211^1 and 211^2 differ from Diet 211 only in containing 40 and 30 mg. of added tryptophane, respectively, instead of 25 mg. 2 equivalents of the indole benzoylaminoacrylic acid were not found to give different results than are shown in this chart for 1 equivalent.

DISCUSSION.

It is clear from Charts I to IV that with the exception of the indole pyruvic acid the derivatives employed had no appreciable influence upon the growth curves. Notwithstanding the close

GROWTH ON INDOLE BUTYRIC ACID, ON INDOLE PROPIONIC ACID AND ON INDOLE ETHYLAMINE.

Rats 69 and 70: Growth on the tryptophane-deficient diet, 211; on Diet 211+2 equivalents of indole butyric acid (2d); on Diet 211, and finally on Diet 211+1 equivalent of tryptophane (a). Rat 85: Dietary changes as above except that the compound tested is indole propionic acid (2 equivalents = 2e). Rat 74: Dietary changes as above except that the compound tested is indole ethylamine hydrochloride (2 equivalents = 2f).

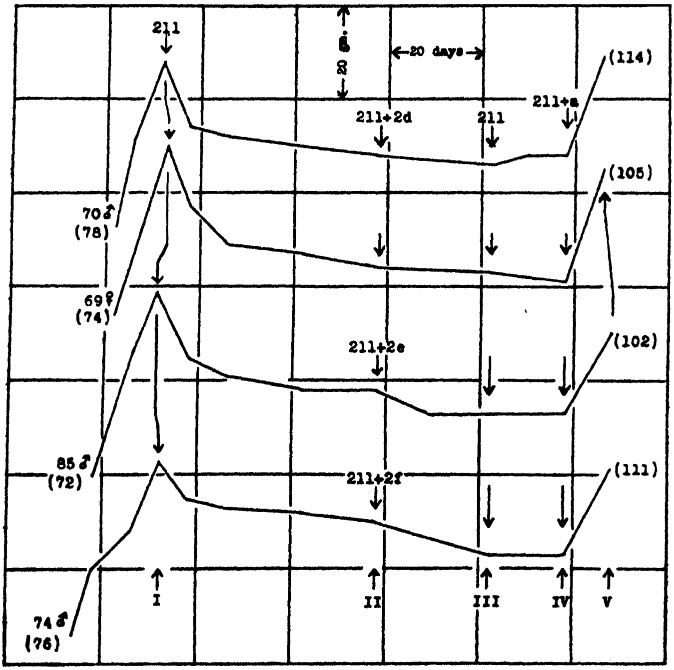


Chart II.

structural relations of the various compounds to tryptophane and the rather superficial chemical alterations necessary to convert them to that product, the organism of the rat did not utilize them for growth. Further brief experiments with indole acetic acid,

indole acetonitrile, and isatin[7] yielded wholly negative results. These results combined with those cited earlier in the paper serve

GROWTH ON INDOLE ETHYL ALCOHOL, ON THE FORMALDEHYDE-TRYPTOPHANE PRODUCT AND ON INDOLE URAMINO PROPIONIC ACID.

Rats 72 and 73: Growth on the tryptophane-deficient diet, 211; on Diet 211 + 2 equivalents of indole ethyl alcohol (2g); on Diet 211; and finally on Diet 211 + 1 equivalent of tryptophane (a). Rats 86 and 89: Dietary changes as above except that the compound tested is the formaldehyde-tryptophane product (2 equivalents = 2h). Rats 75 and 82: Dietary changes as above except that the compound tested is indole uramino propionic acid (2 equivalents = 2i).

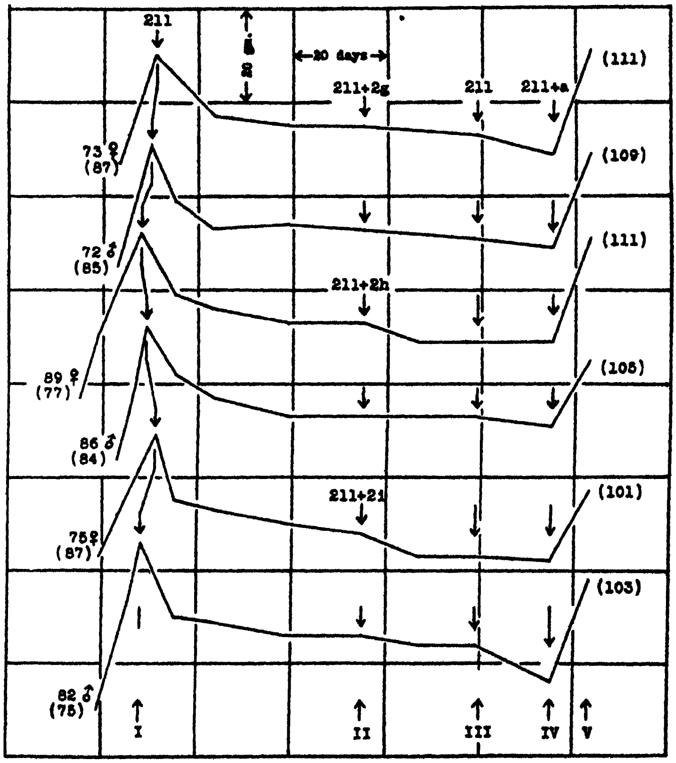


CHART III.

to show how very specific the animal is in its requirements not only for the indole ring—and for an indole ring substituted at

[7] The finding of Whipple and Smith (1928) that tryptophane and isatin both give rise to increased bile salt prompted this experiment.

position 3—but in addition for a side chain of just a certain structure. This exacting type of demand stands in close agreement with that observed in the cases of lysine, histidine, and cystine. For example, the corresponding propionic acid derivative does not

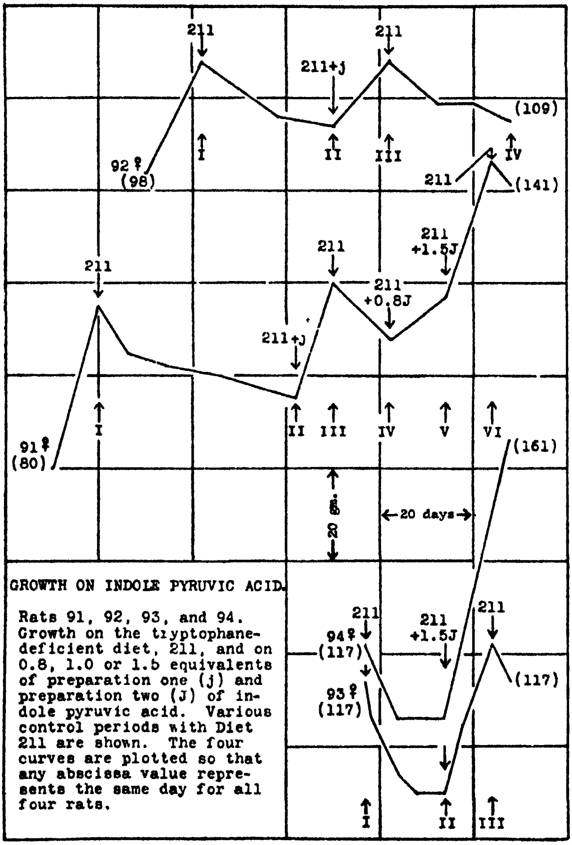


Chart IV.

replace tryptophane, histidine, or cystine in the diet. Apparently few substitutes are acceptable. The failure of indole ethylamine to replace tryptophane stands in agreement in a measure with the finding of Ewins and Laidlaw (1913) that indole ethylamine does not give rise to kynurenic acid; likewise the failure of the

betaine of tryptophane, with the observation of Eagles and Cox (1928) that ergothioneine, the betaine of thiolhistidine, is not appreciably converted to histidine; and finally the failure of the β-indole α-uraminopropionic acid, with the fact that the uramino derivatives of several α-amino acids are excreted unchanged. In general, there is considerable evidence that conjugation of the amino groups of amino acids with alkyl and acyl radicals results in a type of derivative which is quite resistant to chemical alteration in the animal body.

In sharp contrast to the results just discussed stand those secured in experiments with indole pyruvic acid. Chart IV shows the prompt, rapid, and unmistakable body weight increments resulting from the administration of this derivative. Besides the many demonstrations in the literature of the close physiological relations between amino acids and the corresponding pyruvic acids, one recalls here the finding of Harrow and Sherwin (1926) that imidazole pyruvic acid will to a certain extent replace histidine in the diet. The fact that indole pyruvic acid can thus serve in lieu of tryptophane has a bearing on the kynurenic acid formation from indole pyruvic acid in that it emphasizes the possibility already pointed out of the indole pyruvic acid passing through the tryptophane stage in its conversion to the kynurenic acid. No claim can be made, of course, that indole pyruvic acid is necessarily a normal intermediate in tryptophane metabolism. However, this relation may exist, and, in any case, the two substances are certainly physiologically closely connected. Heft and Sherwin[8] have very briefly reported the failure of indole pyruvic acid to effect growth in the absence of tryptophane. The writer offers no explanation of the difference between Heft and Sherwin's finding and his own. It must be kept in mind, of course, that indole pyruvic acid is difficult to prepare in quantity and in a high state of purity.

Criticism of the Method Used.

Sensitiveness.—Chart V shows the growth curves for two sets of rats which were brought into approximate weight maintenance

[8] On p. 155 of Hawk, P. B., and Bergeim, O., Practical physiological chemistry (Philadelphia, 9th edition (1926)), there occurs the sentence: "Heft and Sherwin have found that neither indolepyruvic acid, indolelactic acid nor kynurenic acid can replace tryptophane in the diet."

and then were given in one case 50 mg. (0.2 equivalent) and in the other, 250 mg. (1 equivalent) of tryptophane per 100 gm. of food. There is exhibited a definite growth response to the 50 mg. addi-

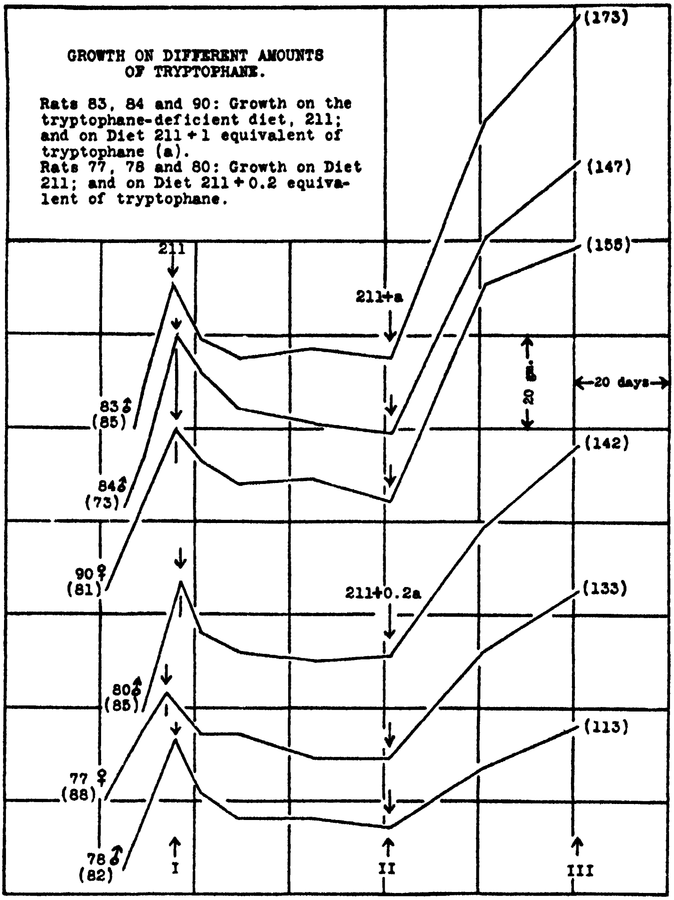


Chart V.

tion and, of course, a still greater and more nearly optimal response to the 250 mg. addition. A larger number of animals fed finely graded dosages, say, between 10 and 100 mg. should give,

when averaged, a fan-shaped set of curves which could be used as the basis for a method of assay of a protein for tryptophane much as Sherman and Woods (1925) have employed for cystine and have suggested for other amino acids. It should be especially pointed out here that of those derivatives fed in 2 equivalent amounts, 10

TABLE II.

Average Daily Food Consumption in Gm.

Rat No.	Period.					
	I	I-II	II-III	III-IV	IV-V	V-VI
58	8.1	3.4	2.8	4.9		
52	7.2	3.4	2.9	5.0		
46	7.6	5.1	4.2	4.0	4.5	7.0
45	8.4	4.5	4.6	4.4	5.0	5.1
70	8.9	4.4	4.1	4.4	7.0	
69	7.5	3.8	3.6	3.7	6.1	
85	7.5	4.5	3.8	4.5	5.5	
74	6.9	5.0	4.2	4.6	5.4	
73	8.1	5.2	4.2	4.2	7.0	
72	8.2	4.2	4.1	4.1	6.0	
89	7.6	4.5	4.3	4.7	6.6	
86	6.8	4.5	4.1	4.4	6.0	
75	6.2	4.8	4.2	4.0	5.1	
82	8.0	4.4	3.6	3.6	7.0	
92	6.4	5.7	5.9	4 6		
91	7.6	5.2	7.3	5.1	5.1	7.3
93		4.1	6.7			
94		5.4	10.1			
83	9.1	5.0	8.1			
84	7.5	5.0	6.7			
90	7.5	4.8	7.5			
80	7.8	4.5	6.5			
77	6.5	5.5	7.3			
78	8.3	4.8	6.1			
44	7.7	5.6	4.6	6.2		

times as many molecules were administered as were given to the rats receiving 50 mg. of tryptophane per 100 gm. of basal diet. In other words, if only 10 per cent of a derivative were converted to tryptophane or to some essential intermediate, the effect would still be easily recognizable. Undoubtedly even a smaller conversion could be detected.

Food Consumption.—Food consumptions were always carefully measured and recorded. The values for these are given in Tables II and III. They show the same general principle that has been emphasized before: Food consumption increases when tryptophane is included in the tryptophane-deficient diet and decreases when this amino acid is omitted. Mitchell (1927) has vigorously attacked all experiments of the type described in this paper on the grounds that a rat may well eat more of a deficient diet after addition of even just a few mg. of the material previously missing, though the supplement is homogeneously dispersed through 100 gm. of the basal diet—because the added material may alter the "flavor, odor, and texture" of the diet better to suit the taste and to whet the appetite of the rat. It is quite difficult to believe

TABLE III.

Average Daily Food Consumption in Gm.

Rat No.	Period.				
	I–III	II–III	III–IV	III–V	V–VI
76	4.1	4.1	4.1 + 2.0		
68	4.1	4.1		4.1	4.1 + 2.0
67	4.6	4.3	4.3 + 2.0		
71	4.6	4.3		4.3	4.3 + 2.0
81	4.0	3.6	3.6 + 2.0		
79	4.0	3.6		3.6	3.6 + 2.0

that such a small amount of one amino acid could exert such an enormous condimental effect when the diet in general already contains some of this amino acid itself and in addition 15 or 20 per cent of other amino acids in the free state—some bitter and some sweet. It is still more difficult to believe that such a principle holds for each of the several different amino acids so far studied, all of which behave similarily in this matter of food consumption. Rose (1928) in replying to Mitchell has adduced logical evidence that an animal under the conditions in question eats more because of a stimulation of general metabolism, which in turn results in appetite increment. The growth is not the result of a condiment, but is a register of increased chemical activity in the cells brought about by the supplying of an indispensable constituent heretofore available in amounts too small for optimum cellular activity.

It was decided to study this question from two angles: (1) Does the rat eat more because of a condimental effect? (2) Is it possible to have test and control animals ingest the same amount of food,

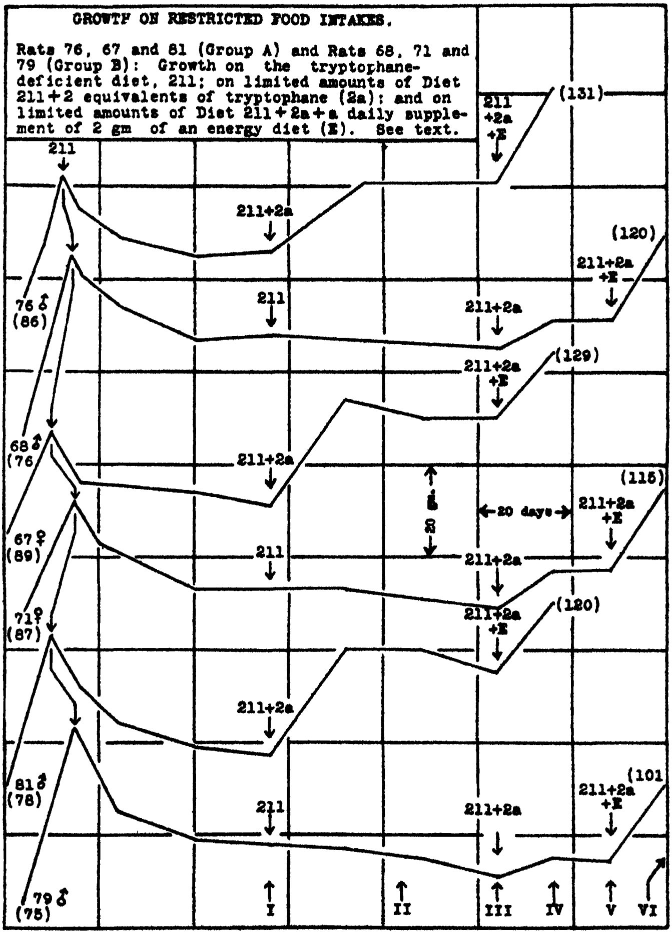


Chart VI.

yet exhibit a difference in growth rates as distinctive and characteristic as is secured with the method now in use? To investigate the first question animals brought into approximate weight

maintenance upon the tryptophane-deficient diet were given daily 25 mg. of tryptophane in the vitamin B mixture apart from the basal diet. Fair growth ensued. The data are not presented here inasmuch as Berg and Rose (1929) have recently demonstrated the same thing in another connection. That the tryptophane given in this manner, *i.e. separately*, and mixed with a small constant amount of yeast concentrate, which the animal eats at once, should affect the "flavor, odor, and texture" of the basal diet which the animal eats *ad libitum* over the whole 24 hours, is beyond credulity.

The second question was studied by bringing a number of rats into approximate weight maintenance on the deficient diet. Six were chosen in three pairs, each pair being of the same sex and exhibiting no greater than a gm. difference in weight (see Chart VI). Three of these (Rats 68, 71, 79, Group B), one from each pair, were continued upon the deficient diet. The other three (Rats 76, 67, 81, Group A), were then restricted to the food consumption of their respective mates, however, with tryptophane added at a 500 mg. level so that it could not possibly be a limiting factor. One-fourth of what one of the pair ate in 4 days was given daily to the second of the pair during the next 4 days (Period I-III). It was estimated that food consumptions were duplicated with an error of 5 per cent or less. The curves show that the Group A rats increased 15 to 20 gm. in weight and then came into another approximate weight maintenance at the higher level. At the end of 48 days, 2 gm. per day of non-protein-containing diet (Diet E) were given additionally to each of these rats (Period III-IV). Their body weights increased immediately, thus showing that the restriction which had been imposed upon them was one primarily of energy. The same type of experiment was repeated with the Group B animals. The food consumption of the last 24 days (Period II-III) of the 48 day experiment was used as a basis for an additional 24 day feeding (Period III-V) of the deficient diet with the 500 mg. supplement of tryptophane. The body weights increased as before—about 5 to 10 gm., to a new maintenance level. And again, addition of the energy diet caused a rapid increase in the body weights (Period V-VI). It appears, therefore, that a rat eating *ad libitum* of a diet limited in one essential factor (as to cause approximate weight maintenance)

ingests an amount of energy which is just about the quantity that can be used advantageously along with the amount available of the limiting factor, and very little more than this. If the limiting factor in the deficient diet were raised to a higher level so that the control animals were growing at a fair rate, then it might be that the test animals restricted to the food consumption of the controls would show a more distinct and continued response to further additions of the factor in question. Even if that were the case, a considerable portion of the method's sensitiveness would be lost in having to distinguish between two rates of growth rather than between growth and maintenance.

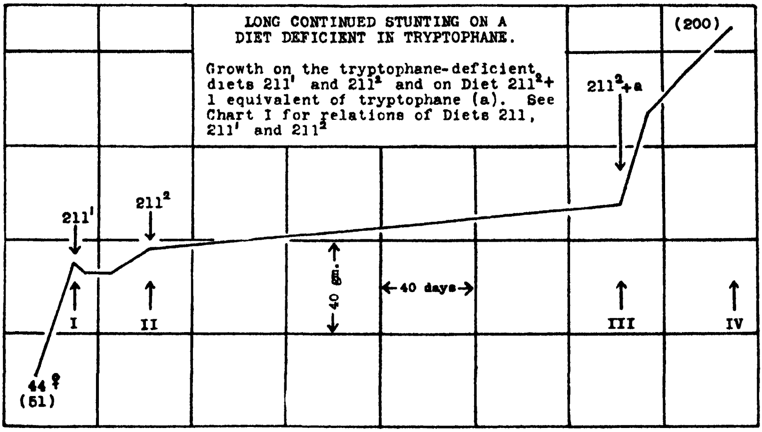


CHART VII

A Case of Long Continued Stunting.

In the course of this study it was debated whether an animal confined for an extremely long period to a tryptophane-deficient diet of the type employed here—containing 15 per cent of free amino acids—would grow at a fair rate after inclusion of more tryptophane. Chart VII shows the growth of Rat 44 under such conditions. The animal's weight at the beginning of the stunting was 100 gm.; 232 days later, it was 125 gm. Inclusion of 250 mg. of tryptophane per 100 gm. of the diet now resulted in a 75 gm. body weight increment in 44 days. During Period II-III (see Chart VII) covering 200 days, the body weight never varied

as much as 2 gm. from a straight line drawn through all of the graphed body weight values. When the animal had reached the weight of 200 gm., a necropsy revealed large stores of fat; the internal organs appeared perfectly normal. The kidneys together weighed 1.3 gm. The two photographs (Fig. 1) depict the appearance of the animal at the end of the stunting period and again at the end of the final growth period. These results are of interest when compared with those secured by Osborne and Mendel (1915) with Rat 2161 which was stunted upon the unhydrolyzed protein zein. The pictures of Rat 2161 are quite comparable to those of Rat 44. Particular attention is called to the hair coats in the two cases. Outstanding harm does not necessarily follow

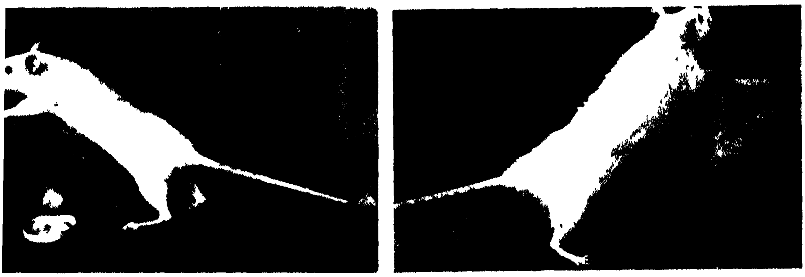


Fig. 1. Photographs of Rat 44 (left) after long continued stunting on a tryptophane-deficient diet, and (right) after a period of growth on the deficient diet supplemented with tryptophane. See points marked III and IV on Chart VII.

long continued stunting under the conditions herein described. In this connection may be recalled Osborne and Mendel's extensive demonstration of the rat's ability to resume normal growth after extremely long periods of stunting.

SUMMARY.

With one exception all the members of a tested series of tryptophane derivatives of physiological significance possesss no appreciable capacity to replace tryptophane in the diet.

Indole pyruvic acid, however, causes resumption of growth in animals deprived of tryptophane.

Data bearing on the relation of growth and food consumption are presented.

An instance of long continued stunting upon a tryptophane-deficient diet is described.

At the XIIIth International Physiological Congress at Boston, August, 1929, Berg, Rose, and Marvel reported a similar result with regard to the indole pyruvic acid; that is, they also have found that indole pyruvic acid serves as a biological substitute for tryptophane.

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ON THE CEREBRONIC ACID FRACTION.

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The established view on the structure of cerebronic acid has recently been put under new scrutiny, and opinions have been expressed against the theory of its being a 2-hydroxypentacosanic acid of the lignoceric series. The history of cerebronic acid is the following.

Thudichum[1] isolated an acid containing 75.94 per cent of carbon and 12.64 per cent of hydrogen and assigned to it the structure C_{18} and the name "neurostearic acid." Thierfelder[2] assigned to the acid the structure of a hydroxypentacosanic acid. Levene, in cooperation with Jacobs,[3] West,[4] and Taylor,[5] has assigned to it the structure of α-hydroxypentacosanic acid of the lignoceric acid series. Levene and Taylor[6] have converted the acid into a tetracosanic acid and the latter into the *d, l*-α-hydroxypentacosanic acid. The tetracosanic acid as well as all the derivatives leading to the α-hydroxypentacosanic acid were compared with corresponding derivatives of lignoceric acid and were found identical with the latter. More recently Klenk[7] advanced the view that cerebronic acid has the structure of α-hydroxytetracosanic acid. Klenk reported the oxidation of cerebronic acid to tricosanic acid and

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more recently, its reduction to lignoceric acid. In their paper on cerebronic acid published in 1922 Levene and Taylor pointed out the possibility of the occurrence of other acids in addition to cerebronic in the fraction composed principally of this acid.

The isolation of such acids, however, has as yet not been accomplished by these authors but the possibility of their occurrence has gained in probability by the findings to be reported in the present communication.

This investigation has been in progress since 1924. Its original aim was to prepare a sufficiently large quantity of α-hydroxypentacosanic acid for the purpose of its resolution, the tetracosanic acid obtained on oxidation of cerebronic acid being used as starting material. The purification of this acid, however, has brought to light the fact that on oxidation of the cerebronic acid used in this work, not a single tetracosanic acid, but a mixture of acids was formed, although the crude oxidation product had the composition and the molecular weight of tetracosanic acid. Considerable quantities of cerebronic acid were required to bring out this fact inasmuch as the separation of the crude material into fractions which differ markedly in composition and molecular weight can be accomplished only by repeated fractional distillations of the esterified acids. An illustration of the method of fractionation is given in the early part of the experimental part and the results of this fractionation are given in Table I. From Table I it is seen that the acids of the molecular weight 365 to 370 on repeated distillation of the esters yielded fractions of molecular weight varying from 351 to 374. Incidentally, it may be mentioned that this fractionation was accomplished without loss of material. On oxidation and subsequent fractionation of the esters, the fractions which in Table I are designated as residues gave acids of molecular weights varying from 360 to 367. From this material a tetracosanic acid of the molecular weight of 367 and with a melting point of 78.5–79.5° was prepared, an acid which is expected to form on oxidation of 2-hydroxypentacosanic acid. It is seen, however, from Table I that the lower fatty acids constitute the greater part of the oxidation product.

From Table I the impression may be gained that the fraction of the lower fatty acids is composed entirely of tricosanic acid. Further scrutiny, however, revealed the fact that these fractions

were mixtures composed of acids of higher and of lower molecular weight than that of tricosanic acid. Thus from benzene solution at room temperature, an acid was obtained of the molecular weight of 361 and from the mother liquors on cooling to 0° an acid separated of molecular weight 355, which melted at 76–77°. By further purification from ether a substance was obtained which corresponded to Klenk's tricosanic acid inasmuch as its molecular weight was 356 and it melted at 77.8–78.6°, Klenk's acid melting at 78.5°. From the mother liquor of this acid a material was obtained of the molecular weight of 350. Klenk's tricosanic acid was found to be an impure substance; by fractional crystallization from benzene it gave a fraction of a higher molecular weight than that of tricosanic acid and from the mother liquor a tricosanic acid was obtained which melted similarly to tricosanic acid obtained from lignoceric; namely, at 76–77°, the mixed melting point of the two having the same value.

Convincing evidence of the impurity of the original tricosanic acid was furnished by the fact that from the mother liquor obtained during its attempted purification, an acid was obtained which had the composition of docosanic acid. This discovery led to a systematic fractionation by distillation of the esters of the acids of the molecular weight of 350 to 356, the results of which are given in Table III. By further purification of these fractions by distillation of their esters and by crystallization from ether, it was possible to show the presence, in the acid which seemed to be pure docosanic acid, of acids of lower molecular weight. The docosanic acid which has reached the highest purity melted at 74.2–75.2°.

It should be emphasized here that the first evidence of the impurity of a higher fatty acid is its inability to crystallize in glistening plates. However, crystallization in glistening plates is not a guarantee of the individuality of the substance.

The question arises as to the significance of the present findings for the problem of the structure of cerebronic acid.

The term "cerebronic acid" was applied to designate the acid component of cerebron, or phrenosin, which in its turn, is the term for that cerebroside which possesses the lowest solubility and the highest dextrorotation. This acid is assumed to have the composition of 2-hydroxypentacosanic acid and is most probably

the parent substance of the tetracosanic acid present in our oxidation product. What then is the origin of the fatty acids of the lower molecular weight? It does not seem probable that they are derived from the same parent substance as the tetracosanic acid. Levene and West showed in 1914 that fatty acids by oxidation with permanganate of their 2-hydroxy derivatives are converted to the extent of 80 per cent into their next lower homologues. Taylor and Levene have shown recently that the same result is obtained with lignoceric acid. From Table I it is seen that the greater part of the acids obtained on oxidation of our material had a molecular

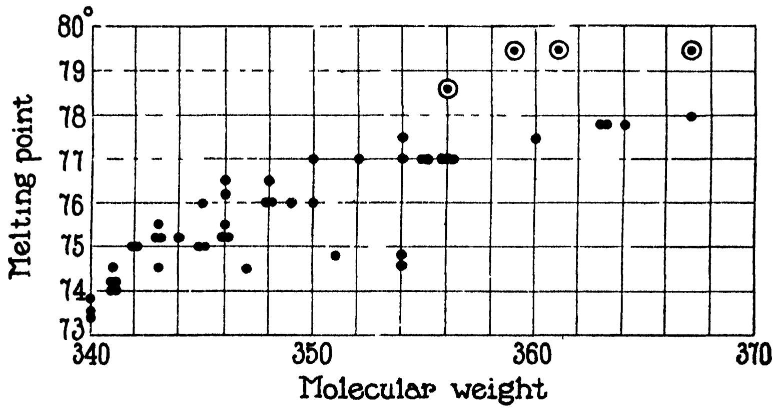

FIG. 1. The melting points and molecular weights of the specimens or fatty acids crystallizing in glistening plates. The points enclosed in circles represent fractions that had been repeatedly crystallized from ether of benzene.

weight lower than tetracosanic acid. Therefore it is warranted to assume that our starting material contained either hydroxy acids of a molecular weight lower than 2-hydroxy-pentacosanic acid or hydroxypentacosanic acids with the hydroxyl group in a position further removed from the carboxyl.

Furthermore, the present observations on the melting points of the tricosanic and docosanic acids which are summed up in Fig. 1 suggest the possibility that these acids do not belong to the lignoceric acid series. The melting points of the impure acids are as high as those of the pure acids of lignoceric acid and sometimes higher than those of the acids of the identical molecular weight

and belonging to the lignoceric acid series. The question as to the details of the structure of the acids of the lower molecular weight cannot yet be answered definitely. Should it be definitely established that these acids are not of the lignoceric acid series, one may be inclined to question into the truth of the assumption that cerebronic acid belongs to the lignoceric acid series. A satisfactory answer to this question will be given after the acids of higher molecular weight present in the oxidation mixture have been studied in greater detail.

The most important conclusion from this work is the conviction that the question of the number of cerebrosides occurring in tissues as well as the problem of the structure of the fatty acids entering in their structure are in need of reinvestigation.

EXPERIMENTAL.

Cerebronic Acid.—The acid, prepared from phrenosin, was in the form of a dull, white powder. It melted at 99.5–100.5° when the temperature of the constantly stirred bath was raised 1° in 7 to 8 seconds. It solidified at 98°. The optical rotation was determined in pyridine solution.

$$[\alpha]_D^{25} = \frac{+0.54° \times 100}{2 \times 7.31} = +3.7°$$

0.1002 gm. substance: 0.2772 gm. CO_2 and 0.1110 gm. H_2O.
0.5614 " " required 7.05 ml. 0.2 N NaOH.[5]

$C_{25}H_{50}O_3$.	Calculated.	C 75.38, H 12.56, mol. wt. 398.
	Found.	" 75.44, " 12.40, " " 398.

Oxidation.—The cerebronic acid was oxidized in lots of 10 gm. The method has been described by Levene and Taylor.[6] For each 10 gm. of the acid, 6.6 gm. of potassium permanganate, dissolved in acetone, were introduced (25 per cent excess). The boiling solution was decolorized in a short time. It was allowed to cool before the precipitated manganese dioxide and soap were filtered off. The material was then suspended in water and treated with sodium bisulfite and hydrochloric acid, with stirring, until all of the manganese dioxide had been destroyed. The acid was then liberated by melting over dilute hydrochloric acid on the water bath.

The fatty acid and soap frequently form a very persistent emul-

sion with the water, which prolongs the time necessary for the liberation of the acid and liquefaction to a clear oil to as much as 48 hours. By alternately heating and cooling to temperatures above and below the melting point of the acid a clear oil is usually obtained, when dealing with small quantities, in the course of 2 to 3 hours.

The acid was crystallized from acetone at 0°. It then usually melted at about 75° and had a molecular weight of 365 to 370. The yield was slightly greater than 8 gm. from 10 gm. of cerebronic acid. About 50 gm. of the oxidation product were prepared.

Fractionation.—Crystallization from an assortment of solvents as well as precipitation of the lithium salts failed to bring about the separation of any fraction that appeared to be a single substance. All fractions separated from solution as dull powders instead of the glistening plates that are characteristic of pure fatty acids.

Distillation however removed the interfering material and gave rise to well crystallized plates with nacreous luster although the distillates usually failed to show sufficient variation in properties. A method by which small fractions of highest and lowest volatility were removed gave the best results.

The procedure followed on two lots of the oxidation product may serve as an example of the methods used in dealing with the remainder. The acid (16.1 gm.) was dissolved in 99.5 per cent alcohol (250 ml.), sulfuric acid (2 ml.) added, and the solution boiled overnight. The ester was crystallized at 0° and recrystallized from alcohol. The washings were then no longer acid to moist litmus paper. The ester (15.7 gm.) was dried and distilled at a pressure of about 0.1 mm.

It was first distilled as completely as possible. The residue (Residue A = 2.2 gm.) was saved. The distillate (13.5 gm.) was again distilled. A small fraction (Distillate B = 1.7 gm.) was first collected and then the remainder distilled in one fraction until a small residue (Residue C = 1.5 gm.) remained in the flask. The middle fraction was distilled in the same manner giving rise to a distillate (Distillate D = 0.8 gm.) and a residue (Residue E = 0.8 gm.). By a further distillation of the middle fraction a distillate (Distillate G = 0.7 gm.), a residue (Residue H = 0.9 gm.), and a middle fraction (Distillate J = 7.1 gm.) were obtained. The boiling point of each fraction was mainly 205–207° with the

bath at 260–265°. The distillates were collected at the rate of 6 to 8 seconds per drop. The pressure was about 0.1 mm.

All of the fractions were saponified and the acids liberated by heating the soaps with dilute hydrochloric acid on the water bath. The acids were crystallized from acetone at 0° after which the constants given in Table I were obtained.

The distillates all crystallized in glistening plates while the residues were without luster. The mother liquors, containing about 2.4 gm. of fatty acids, were saved.

Since unaltered cerebronic acid would be concentrated in the residues, they were combined and subjected to a reoxidation similar to the original treatment of the cerebronic acid. The permanganate was destroyed much more slowly in this instance. The product was little changed. It melted at 75.5–76.5° and solidified at 73°. It was twice crystallized from ether at room temperature and then melted at 76.5–77.5°. It solidified at 73.5°. The yield was 1.8 gm.

TABLE I.

Fractional Distillation of Esters of Crude Oxidation Product.

Fraction.	Yield of acid.	M.p.	Solidification.	Substance.	0.2 N NaOH.	Mol. wt.
	gm.	*°C.*	*°C.*	*gm.*	*ml.*	
Distillate B.	1.5	74–74.6	71.5–71	0.4996	7.06	354
" D.	0.6	74–74.8	71.5–71	0.5004	7.07	354
" G.	0.5	74–74.8	71.5–71	0.5005	7.13	351
" J.	6.5	76–77	73	0.4999	7.02	356
Residue A.	1.5	Indefinite.				
" C.	1.3	75.5–76.5	72.5–72	0.5024	6.72	374
" E.	0.8	75 5–76.5	72–71.5	0.4974	6.68	372
" H.	0.6	76.5–77.5	73	0.5010	6.83	367

0.1000 gm. substance: 0.2868 gm. CO_2 and 0.1145 gm. H_2O.
0.5014 " " required 6.75 ml. 0.2 N NaOH.

$C_{24}H_{48}O_2$. Calculated. C 78.26, H 13.04, mol. wt. 368.
Found. " 78.21, " 12.81, " 371.

It was optically inactive in pyridine solution.

$$[\alpha]_D^{=} \frac{0.00° \times 100}{2 \times 4.63} = 0.0°$$

The ether mother liquors were collected and evaporated and the residue was crystallized from low-boiling gasoline. The acid which separated (1.3 gm.) melted at 75–76° and had a molecular weight of 375.

0.5013 gm. of substance required 6.69 ml. of 0.2 N NaOH.

All of this material was collected (5.8 gm.) and esterified. It was twice distilled as completely as possible at 0.1 mm. and then distilled into four successive fractions. The description of the acids derived from these is given in Table II.

Distillate J (Table I) was crystallized from benzene at room temperature six times. It then melted at 78.5–79.5° and solidified at 76.4–76.2°. The yield was 1.4 gm. and the molecular weight was 361.

TABLE II.

Fractional Distillation of Esters of Reoxidized Residues from Table I.

Fraction.	Yield of acid.	M.p.	Solidification.	Substance.	0.2 N NaOH.	Mol. wt.
	gm.	*°C.*	*°C.*	*gm.*	*ml.*	
Distillate A.	1 2	76 5–77 5	74 5	0.4990	6 93	360
" B.	1 0	76 8–77 8	74	0.4998	6.88	363
" C.	1 2	76 8–77.8	75.5–75	0.5001	6.89	363
" D.	1 0	77–78	75 5–75	0.4999	6.81	367

0.5007 gm. of substance required 6.93 ml. of 0.2 N NaOH.

The mother liquors were crystallized at 0°. The acid which separated (3.3 gm.) melted at 76–77°, solidified at 74–73.6°, and had a molecular weight of 355.

0.5006 gm. of substance required 7.05 ml. of 0.2 N NaOH.

Further crystallization of these fractions failed to bring about a significant change in either the molecular weight or the melting point.

Other lots of material when distilled in the same manner were distributed in a similar series of fractions. In one instance the residues melted at from 77–78.8° and had molecular weights of 357 to 370. The distillates melted at from 76–77° and had molecular weights of 350 to 353.

Crystallization from ether served to separate these distillates into fractions of considerable difference in solubility. Combined

similar distillates (6 gm.) were crystallized from ether at room temperature four times. A yield of 1.3 gm. of acid was obtained which melted at 77.8–78.6° and solidified at 76–75.5°. It was in the form of large glistening plates and was only slightly soluble in cold ether.

0.1004 gm. substance: 0.2873 gm. CO_2 and 0.1140 gm. H_2O.
0.4998 " " required 7.02 ml. 0.2 N NaOH.
$C_{23}H_{46}O_2$. Calculated. C 77.97, H 12.99, mol. wt. 354.
Found. " 78.04, " 12.71, " " 356.

This material seems to be identical with the tricosanic acid described by Klenk[7] which melted at 78.5°. He tested the individuality of his acid by fractional precipitation of the lithium salt. As it is later shown that the substance here described is made up of several acids, the futility of attempting to separate neighboring fatty acids by this means is apparent. Our experience is in agreement with his.

The ether mother liquors were evaporated and the residue was crystallized from low-boiling gasoline. The fine glistening plates which separated, melted at 75–76° and solidified at 73.5–73.° The molecular weight was 350.

0.4992 gm. of substance required 7.13 ml. of 0.2 N NaOH.

Repeated crystallization of this material was without further effect.

Several fractions of the large plates were combined. The melting point range was from 77–78.8° and the molecular weights were 353 to 357. This material (6.3 gm.) was crystallized six times from benzene at room temperature. The yield was 2.1 gm. and the molecular weight was 359. The melting point was 78.5–79.5° with resolidification at 76.2–76°.

0.4989 gm. of substance required 6.95 ml. of 0.2 N NaOH.

From the mother liquors at 0°, 2.8 gm. of plates of melting point 76–77° separated. Solidification took place at 74.2–73.8°. The molecular weight was 355.

0.5002 gm. of substance required 7.05 ml. of 0.2 N NaOH.

A specimen of acid was obtained by the distillation of the ester from 10 gm. of large well crystallized plates which melted at 76.5–77.5° and solidified at 74.5°. The molecular weight was 354.

0.4997 gm. of substance required 7.06 ml. of 0.2 N NaOH.

A melting point determination was made on this material together with the isotricosanic acid from lignoceric acid described by Taylor and Levene[5] in the proportion 1:1. The isotricosanic acid melted at 76–77° and solidified at 74.5–74°. The melting point of the mixture was 76–77° with resolidification at 74°. The acid

TABLE III.

Fractional Distillation of Esters of Collected Acids of Molecular Weight 350 to 355.

Fraction.	Yield of acid.	M.p.	Solidification.	Substance.	0.2 N NaOH.	Mol. wt.
	gm.	*°C.*	*°C.*	*gm.*	*ml.*	
Distillate A.	1.7	73 5–74 5	72 5–72	0.4997	7.20	347
" C.	1 8	74–75	72.5–72	0.5006	7.25	345
" E.	1.9	74–75	72.5–72	0.5008	7.25	345
" H.	3.1	74 5–75 5	73–72.5	0.5000	7.23	346
" J.	1.6	75–76	74–73	0.5007	7.19	348
Residue B.	1.1	74.5–75.5	73–72	0.5004	6.76	370
" D.	1.5	76–77	74.5	0.4999	7.02	356
" G.	1.1	76–77	74 5	0.5003	7.03	356
" K.	1.4	76–77	74.5	0.5002	7.05	355

TABLE IV.

Fractional Distillation of Distillates of Table III Together with Distillate A (P. 33).

Distillate.	Yield of acid.	M.p.	Solidification.	Substance.	0.2 N NaOH.	Mol. wt.
	gm.	*°C.*	*°C.*	*gm.*	*ml.*	
A	1.4	72.4–73 4	71.2–70.6	0.5008	7.37	340
B	1.4	72.5–73.5	71.5–71	0.5001	7.36	340
C	2.4	72.8–73.8	71.5–71	0.4990	7.33	340
D	3.2	74–75	72	0.5000	7.32	342

from cerebronic acid became a part of the material described in Tables III, IV, and V.

While most of these fractions have molecular weights nearly in agreement with that of a tricosanic acid, the correspondence is not satisfactory. The presence of very small amounts of ester or soap may explain high figures, but only the admixture of acid of lower equivalent weight can account for the increased consumption of alkali. The possibility was early recognized that this material

might be made up of a mixture of several fatty acids. Evidence in favor of this was obtained on distilling the ethyl esters of about 25 gm. of residues that had accumulated by combining mother liquors and allowing them to evaporate. The ester was prepared in the usual manner, boiled in alcoholic solution with blood charcoal and crystallized at 0°. It was then distilled at a pressure of 0.1 mm. The distillate which boiled at 205–227° with the bath at 270–295° weighed 14.5 gm. This was redistilled into two fractions, the first of which (Distillate A = 4.6 gm.) boiled at 205–208° and the second (Distillate B = 9.0 gm.) at 209–214° both with the bath at 260°. They were saponified. Distillate A melted at 71–72° and resolidified at 70.5–70°.

0.5010 gm. substance required 7.26 ml. 0.2 N NaOH.

$C_{22}H_{44}O_2$. Calculated. Mol. wt. 340. Found. Mol. wt. 345.

After two crystallizations from ether at 0°, about a third of the material was in the mother liquor. The melting point was then 73–74° with resolidification at 71.5°. The molecular weight was still 345.

0.5008 gm. substance required 7.26 ml. 0.2 N NaOH.

Distillate B melted at 73–74° and had a molecular weight of 354.

0.5009 gm. substance required 7.07 ml. 0.2 N NaOH.

Neither fraction crystallized in lustrous plates.

All fractions of molecular weight 350 to 355 were then combined. The melting points ranged from 74.6–78°. The ethyl esters (17.5 gm.) to which they gave rise were distilled into nine fractions by the procedure already described. They all boiled between 210 and 215° at a pressure of 0.1 mm. The constants of the acids are given in Table III.

These distillates together with Distillate A (mol. wt. 345), see above, were collected and esterified for redistillation. The ester was first distilled as completely as possible and then fractionated as before. The residues were held and the distillates saponified. The acids from them are described in Table IV.

These fractions were united and crystallized from 10 parts of ether at 0°. Two crystallizations brought the melting point to 74–75°, with resolidification at 72.5°, after which it could be changed no further. The yield of large glistening plates was

7 gm. The docosanic acid from lignoceric acid, described by Meyer, Brod, and Soyka[8] melted at 75°.

0.1000 gm. substance: 0.2855 gm. CO_2 and 0.1171 gm. H_2O.
0.5000 " " required 7.31 ml. 0.2 N NaOH.
$C_{22}H_{44}O_2$. Calculated. C 77.65, H 12.94, mol. wt. 340.
Found. " 77.86, " 13.10, " " 342.

The mother liquors of the individual fractions and from the first crystallization from ether were combined and evaporated and the residue was resaponified to destroy any ester present. The free acid was crystallized from acetone at 0° and then melted at 67–68°. The molecular weight was 330.

0.5012 gm. of substance required 7.60 ml. of 0.2 N NaOH.

TABLE V.

Fractionation of Distillate B (P. 33) Together with Residues Corresponding to Distillates of Table IV.

Fraction.	Yield of acid.	M p.	Solidification.	Substance.	0.2 N NaOH.	Mol. wt.
	gm.	*°C.*	*°C.*	*gm.*	*ml.*	
Distillate A.	2.1	74.2–75.2	72.5–72	0.4997	7.22	346
" B.	1.7	74.2–75.2	72.5–72	0.5000	7.27	344
" C.	3.2	74.2–75.2	72.5–72	0.4995	7.22	346
" D.	3.3	75–76	73–72.5	0.4999	7.18	348
Residue.	2 4	76–77	74.5	0.5007	7.11	352

The acid (Distillate B) of molecular weight 354 and m.p. 73–74°, p. 33, obtained from the collected residues, was combined with the residues of the above distillation and esterified. The ester (19.8 gm.) was twice distilled as completely as possible. It distilled very constantly at 206° with the bath at 275°. The pressure was 0.1 mm. It was then separated into five fractions by removing successive portions of high- and low-boiling material.

The residues, excepting the last, were held for further distillation. The four distillates and the last residue were saponified. The acids, which crystallized in glistening plates, are described in Table V.

The combined distillates, together with the material in the mother liquors, were esterified and distilled twice, only a small

[8] Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, **34**, 1113 (1913).

residue being left in the flask each time. The second distillate was collected in two fractions. The acid (Distillate A = 6.7 gm.) from the first melted at 73.5–74.5°, solidified at 71.5°, and had a molecular weight of 343. The acid (Distillate B = 2.4 gm.) from the higher fraction of the distillate melted at 75–76°, solidified at 73–72.5°, and had a molecular weight of 349.

Distillate A.—0.5007 gm. substance required 7.30 ml. 0.2 N NaOH.
" *B.*—0.4990 " " " 7.15 " 0.2 " "

Although the acid (Distillate A) was apparently nearly pure C_{22} acid, fractionation of the ester brought about considerable variation in the acids isolated. The range of molecular weight was 342 to 351. All material approximating C_{22} acid in composition

TABLE VI.

Fractional Distillation of Apparently Pure Docosanic Acid.

Fraction.	Yield of acid.	M.p.	Solidification.	Substance.	0.2 N NaOH.	Mol. wt.
	gm.	*°C.*	*°C.*	*gm.*	*ml.*	
Distillate A.	1.7	73–74	71.4–71	0.4999	7.33	341
" B.	1 6	73.2–74.2	72–71.6	0.5003	7.34	341
" C.	1 8	73 2–74.2	71.5–71	0.5005	7.34	341
" D.	1 7	73 5–74.5	72–71.6	0.5005	7.33	341
" E.	1.9	75–76	73–72 6	0 4998	7.25	345
" G.	2.1	75 5–76.5	73.5	0.5003	7.19	348
Residue H.		76–77	74 5	0.4995	7.13	350

was therefore collected and the ester distilled in the manner already described. The residues were united and distilled into three fractions (Distillates E and G and Residue H). The constants of the recovered acids are given in Table VI. All fractions crystallized from acetone in lustrous plates.

On crystallization from ether, the first four fractions (united) gave rise to 5.1 gm. of acid with a molecular weight of 341. It melted at 74–75°.

0.4970 gm. of substance required 7.29 ml. of 0.2 N NaOH.

From the mother liquors, two fractions were obtained. The higher fraction (1.0 gm.) melted at 71.5–72.5° and the lower (0.6 gm.) at 71–72°. The molecular weights were 332 and 330 respectively.

0.4999 gm. substance required 7.54 ml. 0.2 N NaOH.
0.5025 " " " 7.62 " 0.2 " "

The above 5.1 gm. of acid were esterified and distilled into four successive fractions. The residue (0.7 gm.) had a molecular weight of 348. The acids from the distillates melted at from 75–75.6° and had molecular weights of 340, 342, and 344. The latter were united and thrice crystallized from ether at 0°. The least soluble fraction (3.4 gm.) then melted at 74.2–75.2° and had a molecular weight of 343.

0.4987 gm. of substance required 7.26 ml. of 0.2 N NaOH.

It was finally crystallized three times from 250 ml. of ether at 0°. The mother liquors were evaporated separately and the residues crystallized from acetone. The fractions obtained are described in Table VII.

Fractionations designed to isolate the higher fatty acids present in the mixture have not been as extensively carried out as in the

TABLE VII.

Fractional Crystallization of Apparently Pure Docosanic Acid from Ether.

Fraction.	Yield of acid	M p.	Solidification	Substance	0 2 N NaOH	Mol wt
	gm.	°*C.*	°*C.*	*gm.*	*ml.*	
A	0 7	74 2–75 2	72	0 5009	7 30	343
B	0 7	74 5–75 5	73	0 5002	7 29	343
C	0 8	75 2–76 2	73 5	0 4990	7 22	346
D (top).	1.1	75 5–76 5	74 5–74	0 4996	7 22	346

case of the lower members but the presence of acid of molecular weight greater than that of $C_{23}H_{46}O_2$ (354) has been established.

By similar methods of distillation a sample has been obtained with a molecular weight of 364. It melted at 76.8–77.8°.

0.5007 gm. of substance required 6.88 ml. of 0.2 N NaOH.

Another specimen obtained by repeated crystallization from ether melted at 78.5–79.5°.

0.1000 gm. substance: 0.2869 gm CO_2 and 0.1180 gm. H_2O.
0.4981 " " required 6.78 ml. 0.2 N NaOH.

$C_{24}H_{48}O_2$	Calculated.	C 78.26, H 13.04, mol. wt. 368.
	Found.	" 78.24, " 13.20, " " 367.

Both of these specimens crystallized in lustrous plates.

From about 50 gm. of the oxidation product of cerebronic acid, the yield of acid crystallizing in glistening plates was 40 gm.

The range of molecular weight was mainly between 340 and 368. About 2 gm. had a molecular weight of 330 to 332. The high boiling residues apparently contained cerebronic acid or its condensation products as they were optically active. They crystallized as dull powders. The molecular weight of a specimen that melted at 79–81° was 394.

0.5004 gm. of substance required 6.35 ml. of 0.2 N NaOH.

The optical rotation was taken in pyridine solution.

$$[\alpha]_D^{20} = \frac{+0.19° \times 100}{2 \times 6.00} = +1.6°$$

All of the melting points are corrected.

SUMMARY.

1. Cerebronic acid from phrenosin has been degraded by oxidation and a study has been made of the resulting unsubstituted fatty acids.

2. An oxidation product, uncontaminated by cerebronic acid and crystallizing in lustrous plates, has been obtained. The analytical figures indicate that it is a tricosanic acid.

3. Contrary to the previous findings of various investigators, the acid has been fractionated giving rise to several acids of higher and lower molecular weight. The individuality of these fractions has not been established.

4. The origin of these acids and the bearing of their presence on the question of the structure of cerebronic acid has been discussed.

STUDIES IN POLYMERIZATION AND CONDENSATION.

V. CONDENSATION PRODUCTS OF METHYL-CYCLODIHYDROXYACETONE.

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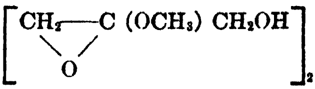
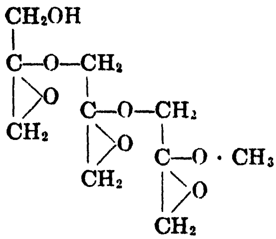
In a previous communication[1] we have shown that dihydroxyacetone, which is normally supposed to exist in dimeric form, either spontaneously or through the action of heat readily condenses through loss of water into substances composed of 2 or 3 molecules of the parent substance. To the methylcycloacetal of dihydroxyacetone also the dimeric form is attributed by Fischer and Taube.



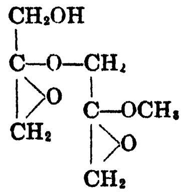
In this substance the reactive group is stabilized by the introduction of the methyl group. It therefore was not to be expected that it could condense in a manner similar to the free dihydroxyacetone; on the other hand, its ready polymerization into the dimeric form permitted the expectation that under suitable conditions the polymerization may proceed to formation of products of higher molecular weight than the dimeric form. In order to test this possibility methylcycloacetal was heated at a temperature slightly above its melting point and the product was analyzed. From the reaction mixture, to our surprise, a condensation product was obtained which had the composition of [di(dihydroxyacetonyl)]-methyldihydroxyacetone and which therefore had the following structure.

[1] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **78**, 23 (1928)



The formation of this derivative indicates the great instability of the glucosidic union in the methylcycloacetal of dihydroxyacetone. To test this assumption the methylcycloacetal was heated to the temperature of its melting point and the volatile products formed during the process were collected and analyzed and they were found to consist mainly of methanol. The great instability of the glucosidic union was further demonstrated by the fact that the cycloacetal on heating with *p*-nitrophenylhydrazine in a 60 per cent solution of acetic acid readily formed an osazone.

Cyclomethylacetal of dihydroxyacetone had been prepared previously by Fischer and Taube from freshly distilled monomeric dihydroxyacetone which was acted upon by methanol in the presence of hydrochloric acid. We found no difficulty in preparing the substance from the commercial dihydroxyacetone[3] in the presence of hydrogen chloride; in other words, by the classical method for the synthesis of glucosides. In the reaction product, in addition to some free dihydroxyacetone and cyclomethylacetal of dihydroxyacetone, a second condensation product of cyclomethylacetal was found; namely, the dihydroxyacetonyl (methylcyclo) dihydroxyacetone of the following composition.



[3] Our acknowledgement is due to H. A. Metz and Company who very generously supplied us with the dihydroxyacetone used in this investigation.

This product was isolated as the acetyl derivative. *Thus, it seems that cyclomethylacetal of dihydroxyacetone readily forms condensation products analogous to those of the free dihydroxyacetone.* It must be remarked, however, that regarding the second condensation product, there exists no rigid proof that the dihydroxyacetonyldihydroxyacetone had not existed preformed in the crude dihydroxyacetone. On the other hand, the formation of the trimolecular complex undoubtedly passes through the stage of the dimolecular form. Hence, there is an equal probability that the substance is formed from the methylcycloacetal.

EXPERIMENTAL.

Methylcyclodihydroxyacetone.—This compound had been prepared by Fischer and Taube[3] from purified monomeric dihydroxyacetone. It was found that the methylcycloacetal could be more conveniently prepared from commercial dimeric dihydroxyacetone in the following manner: 60 gm. of dihydroxyacetone were dissolved in 400 cc. of dry methyl alcohol containing 0.5 per cent of hydrogen chloride. The faintly turbid solution was clarified by means of a little charcoal and allowed to stand overnight at room temperature. The hydrogen chloride was removed by shaking with silver carbonate, a little charcoal was added, and the mixture was filtered. The clear alcoholic solution was concentrated in an evaporating dish by blowing a rapid stream of warm air over the surface of the solution (in a hood). The solvent was occasionally removed under reduced pressure at a low temperature. On scratching the walls of the evaporating dish with a glass rod, crystallization soon set in. After standing in a desiccator overnight, at zero degrees, the crystals were filtered off and sucked as dry as possible. 24 gm. of crude methylcyclodihydroxyacetone were obtained. Filtrate I containing some unchanged dihydroxyacetone was again dissolved in 120 cc. of dry methyl alcohol containing 0.5 per cent of hydrogen chloride and the process described above was repeated. Another 5 gm. of crude methylcyclodihydroxyacetone were obtained on seeding the concentrate. These crystals were washed on the suction funnel with ethyl acetate. On allowing the filtrate to stand at zero degrees another 5 gm. or a total of 34 gm. of crude methylcyclodihydroxyacetone

[3] Fischer, H. O. L., and Taube, C., *Ber. chem. Ges.*, **60**, 1706 (1927).

were obtained. This material was recrystallized from 80 cc. of hot ethyl acetate and yielded 23 gm. of crystalline substance which melted at 127–128°. A part of this methylcycloacetal was dried at 50–70° under 2 to 5 mm. pressure. This material was extracted with 4 times its weight of ethyl acetate in which it was not completely soluble and filtered with suction. From the somewhat concentrated filtrate, methylcyclodihydroxyacetone crystallized on cooling. It melted at 130–131°. It had the following composition.

5.510 mg. substance: 9 340 mg. CO_2 and 3.825 mg. H_2O.

$C_4H_8O_3$ (104.1).	Calculated.	C 46.13,	H 7.74.
	Found.	" 46.22,	" 7.76.

The less soluble fraction had a slightly higher melting point (136°). The substance, however, also analyzed for methylcyclodihydroxyacetone.

5 245 mg. substance: 8.855 mg. CO_2 and 3.620 mg. H_2O.

$C_4H_8O_3$ (104.1).	Calculated.	C 46.13,	H 7.74.
	Found.	" 46.03,	" 7.72.

Condensation of Methylcyclodihydroxyacetone.—4.0 gm. of pure methylcyclodihydroxyacetone (m.p. = 130–131°) were kept for 3 hours at a temperature of 138–140° in a sealed tube. The temperature was gradually lowered to 100° over a period of 4 hours and kept at this temperature for another 16 hours. A substance of crystalline appearance deposited on the glass wall of the tube. The contents of the tube were treated with a mixture of equal parts of methyl alcohol and ether and transferred to a Buchner funnel. The material was triturated with ether and alcohol (1:1). It was slightly soluble in pyridine and practically insoluble in water, alcohol, acetone, ether, benzene, camphor, and ethyl acetate. It reduced hot Fehling's solution strongly. The substance decomposed around 300° and analyzed for [di(dihydroxyacetonyl)]-methyldihydroxyacetone.

5.455 mg. substance: 9.660 mg. CO_2 and 3.095 mg. H_2O.

$C_{10}H_{16}O_7$ (248.18).	Calculated.	C 48.36,	H 6.50.
	Found.	" 48 28,	" 6.34.

Cleavage of Methyl Alcohol from Methylcyclodihydroxyacetone.—5 gm. of methyldihydroxyacetone were kept in a distilling flask the

bulb of which had the shape of an Erlenmeyer flask. The flask was partly immersed in a glycerol bath and the temperature maintained at 128–133° for 7 hours. The receiver was kept in an efficient cooling mixture (solid CO_2 and alcohol) and was provided with a side tube which was protected from the atmospheric moisture by a calcium chloride tube. During a short time at the beginning of heating, part of the substance sublimed, depositing on the cooler part of the flask. After heating the substance 7 hours at practically its melting point, it had lost 0.5 gm. in weight. About 0.5 cc. of a colorless liquid was collected in the receiver. It had the composition of methyl alcohol.

2.990 mg. substance: 4.155 mg. CO_2 and 3.330 mg. H_2O.

CH_4O (32.03).	Calculated.	C 37.48,	H 12.59.
	Found.	" 37.89,	" 12.46.

This distillate gave a pink color with Schiff's reagent and also a small amount of a red precipitate with *p*-nitrophenylhydrazine. This is perhaps due to the presence of a small amount of methylglyoxal formed during the decomposition of the methylcyclodihydroxyacetone.

p-Nitrophenylhydrazine and Methyldihydroxyacetone.—Inasmuch as the methyl group of methylcyclodihydroxyacetone is readily hydrolyzed, treatment with *p*-nitrophenylhydrazine in 60 per cent acetic acid yields the corresponding osazone of dihydroxyacetone. For comparison, a sample of this osazone was prepared directly from dihydroxyacetone. Both osazones decomposed without melting at 260°.

The osazone prepared from methyldihydroxyacetone analyzed as follows:

4.015 mg. substance: 7.450 mg. CO_2 and 1.350 mg. H_2O.
4.070 " " : 0.835 cc. N_2 at 27° and 759 mm.

$C_{15}N_{14}N_6O_5$. (358.25).	Calculated.	C 50.27,	H 3.96,	N 23.47.
	Found.	" 50.37,	" 3.76,	" 23.30.

Mother Liquor of Methylcyclodihydroxyacetone (Filtrate I).

Filtrate I obtained as the mother liquor of the crude methylcyclodihydroxyacetone is a mixture of several substances. The presence of the following substances was made evident: dihydroxy-

acetone, two isomeric methylcyclodihydroxyacetones, and a condensation product, namely methyldihydroxyacetonyldihydroxyacetone. The substances were isolated in form of their acetyl compounds. The first mentioned was also isolated in the free state.

Dihydroxyacetone.—This compound may be obtained directly from the mother liquor mentioned above by distillation under greatly reduced pressure. Thus at the temperature of 92–96° at 0.1 to 0.2 mm. a substance distilled which crystallized in the receiver. It reduced cold Fehling's solution strongly and had a sweet taste.

Acetylation of Mother Liquor. Diacetyldihydroxyacetone.—The mother liquor that had been obtained on methylation of 85 gm. of dihydroxyacetone gave on addition of about 150 cc. of ethyl acetate an oily layer which settled to the bottom and was estimated at about 4 cc. This somewhat turbid fraction was completely freed from solvent by distillation under reduced pressure and the remaining residue was acetylated with a mixture of 15 cc. of acetic anhydride and 15 cc. of dry pyridine which was allowed to stand overnight. The small amount of precipitate that was formed was removed by filtration. It contained 43 per cent of ash. The filtrate on concentration and standing deposited a small amount of a crystalline material. After trituration with ethyl acetate and hot benzene the substance sintered at 180° but had not melted at 300° though it turned black. It is probably a mixture of acetylated condensation products of dihydroxyacetone. It contained 44.2 per cent of carbon and 6.0 per cent of hydrogen. The remainder of the acetylation mixture yielded on distillation under greatly reduced pressure a substance that distilled at 83° under 0.3 mm. pressure. It crystallized in the receiving flask on cooling. The substance analyzed for diacetyldihydroxyacetone.

6.055 mg. substance: 10.745 mg. CO_2 and 3.375 mg. H_2O.

$C_7H_{10}O_5$ (174.11).	Calculated.	C 48.27, H 5.79.
	Found.	" 48.39, " 6.23.

Acetylmethylcyclodihydroxyacetone.—The dark distillation residue obtained from the foregoing distillation was dissolved in ethyl acetate. On standing overnight at zero degrees, a dark crystalline material deposited. Several recrystallizations from ethyl acetate

and from a mixture of methyl alcohol and ethyl acetate, a little charcoal being used each time, yielded a white crystalline substance. Its melting point was thus raised to 135°. It analyzed for acetylmethylcyclodihydroxyacetone.

3.480 mg. substance: 6.320 mg. CO_2 and 2.250 mg. H_2O.

$C_6H_{10}O_4$ (146.11).	Calculated.	C 49.30, H 6.90.
	Found.	" 49.52, " 7.23.

A mixture of this substance with acetylmethylcyclodihydroxyacetone prepared from methylcyclodihydroxyacetone did not show an appreciable melting point depression.

Acetyldihydroxyacetonyl(Methylcyclo)Dihydroxyacetone.—The ethyl acetate solution of Filtrate I, obtained after removing the oily bottom layer as described above, was concentrated under diminished pressure. 29 gm. of a clear lemon-colored syrup resulted. This was dissolved in a mixture of 100 cc. of dry pyridine and 100 cc. of acetic anhydride (under cooling with ice water). After standing at 13° overnight a small amount of substance had crystallized out. It was separated, washed with hot methyl alcohol, triturated twice with warm ethyl acetate and finally washed with a little ether. The substance melted at 184° and analyzed for acetyldihydroxyacetonyl methyldihydroxyacetone.

4.960 mg. substance: 9 075 mg. CO_2 and 2.855 mg. H_2O.

$C_9H_{14}O_6$ (218.15)	Calculated.	C 49.53, H 6.47.
	Found.	" 49.89, " 6.44.

Somewhat more of this substance was obtained from the acetylation mixture (Filtrate II) on concentrating under diminished pressure. The concentrate was kept at zero degrees overnight. A crystalline mixture resulted which was filtered off with suction. The crystalline material was then triturated with the following solvents.

Extract A.—Three times with 15 cc. of acetone.
" *B.*—Twice with 15 cc. of ethyl acetate.
" *C.*—Twice with 10 cc. of hot methyl alcohol.

The remaining insoluble part was treated with a large amount of hot benzene. On cooling, a crystalline substance was secured

which melted at 181–182°. It analyzed for acetyldihydroxyacetonyl methyldihydroxyacetone.

4.505 mg. substance: 8.175 mg. CO_2 and 2.710 mg. H_2O.
0 0864 gm " : 0.1032 gm. AgI.

$C_9H_{14}O_6$ (218.15).	Calculated.	C 49 53,	H 6.47,	OCH_3 14.23.
	Found.	" 49.48,	" 6.73,	" 15.76.

Molecular Weight Determination.—The molecular weight determination on this substance by cryoscopy showed that about 50 per cent of the molecules are in a dimeric condition. Solvent 39.391 gm. of bromoform (c = 143).

Weight of substance. *gm*	Freezing point depression.	Molecular weight.
0 0542	0 061°	323
0 1232	0 140°	320
0 1881	0 212°	322

Molecular weight calculated (monomeric) 218.15. The molecular weight determination by the Smith and Young[4] modification of Rast's method gave a value of 319.

Acetyl Determination.—0.0850 gm. of substance was allowed to stand with 40.0 cc. of 0.1 N NaOH overnight. 35.7 cc. of 0.1 N HCl were required for neutralization.

CH_3CO. Calculated. 19.73. Found. 21.76.

Crystalline Substance with Melting Point of 112°.—Extract A, acetone, Extract B, ethyl acetate, and Extract C, methyl alcohol, on concentration under reduced pressure and cooling yielded a crystalline substance which sintered at 105° and melted at 112°. Its composition was practically the same as that of acetylmethylcyclodihydroxyacetone, of which it is probably an impure sample.

Examination of Mother Liquor of Acetylation Products (Filtrate III).

The mother liquor of the above crystalline fraction was subjected to distillation under reduced pressure. The following three fractions were obtained.

Fraction	I.	Boiling from	85– 95°	at 0.4 to 0.5 mm.
"	II.	" "	110–140°	" 0.6 " 0.4 "
"	III.	" "	145–175°	" 0.6 " 0.4 "

[4] Smith, J. H. C., and Young, W. G., *J. Biol. Chem.*, **75**, 289 (1927).

Fraction I.—This fraction, a liquid, analyzed as acetylmethyldihydroxyacetone.

7.295 mg. substance: 13.225 mg. CO_2 and 4.555 mg. H_2O.
$C_6H_{10}O_4$ (146.11). Calculated. C 49.30, H 6.90, OCH_3 21.22.
Found. " 49.43, " 6.98, " 23.24.

In distinction to the crystalline acetylmethyldihydroxyacetone this compound reduced Fehling's solution very strongly. This substance, probably an isomer of the former, is therefore methoxycycloacetyldihydroxyacetone; *i.e.*, the methoxyl group is attached to an end carbon atom while the acetyl group is combined in ester form with the central or acetal-forming carbon atom of dihydroxyacetone.

Molecular Weight Determination by Cryoscopy.—The molecular weight, determined in benzene, gave a figure which lies between the values calculated for the mono- and dimeric forms. This compound is therefore less polymerized than its isomeric form. Solvent: 12.3843 gm. benzene (k = 5.07).

Weight of substance. *gm*	Temperature depression.	Molecular weight.
0 1102	0 206°	219
0 2750	0 516°	219
0 4602	0.862°	219

Molecular weight calculated ($C_6H_{10}O_4$) monomeric 146.11.

Fraction II.—This fraction crystallized for the greater part in the receiver. The recrystallized material melted at 103–106° and analyzed in a manner similar to the preceding fraction but it did not reduce Fehling's solution.

Fraction III.—Part of the material in the receiver crystallized. A little ether was added and after cooling, the crystals were sucked off. They were dissolved in hot methyl alcohol and the solution was filtered. On cooling, a crystalline material was obtained which softened at 115° and melted at 140°. From the filtrate, after slight concentration, another small crop of crystals was secured which melted from 120–130°. The two fractions were united and recrystallized from ethyl acetate. The material softened at 115° but melted completely at 155°. The substance is probably contaminated with a small amount of a higher condensation product along with a larger part of acetylmethyldihydroxyacetone for which it analyzed.

4.755 mg. substance: 8.620 mg. CO_2 and 6.74 mg. H_2O.
$C_6H_{10}O_4$. Calculated. C 49.30, H 6.90.
Found. " 49.43, " 6.74.

THE CARBOHYDRATE GROUP OF OVOMUCOID.

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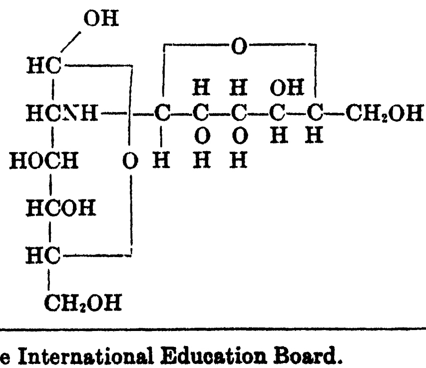
The problem of the presence of a carbohydrate group in proteins and particularly in ovalbumin is of very old standing and has never been definitely settled. A review of the literature on the subject was given in the monograph on "Hexosamines and Mucoproteins"[1] and more recently by Fränkel and Jellinek.[2] The occasion for the latter review was a report of the isolation from coagulated egg white and from yolk proteins of a new polysaccharide which was composed of glucosamine and mannose. The discovery by these authors is very significant for the reason that never before has mannose been found among the constituents of animal tissues. The authors attributed to the substance the structure of a disaccharide in which the aldehydic group of the mannose was attached to the amino group of glucosamine in the following manner, or rather of a polymer of the latter inasmuch as the substance did not reduce Fehling's solution whereas the disaccharide should do so.



[*] Fellow of the International Education Board.

[1] Levene, P. A., Hexosamines and mucoproteins, New York, pt. 3, sect. B (1925).

[2] Fränkel, S., and Jellinek, C., *Biochem. Z.*, **185**, 392 (1927).

During Professor Fränkel's visit to this country, he suggested to one of us that we undertake the further work on the details of the structure of the new polysaccharide. We were interested in doing so for the reason that the nature of the carbohydrate of such glucosidoproteins, which are not true mucoproteins, is as yet not known.

One of the principal difficulties in the preparation of the prosthetic group of the conjugated proteins is the removal of all material giving the biuret test. We have found that all such material can be easily removed by means of mercuric sulfate. However, in order that this procedure may be successful, one condition must be fulfilled; namely, the prosthetic group prior to its purification by mercuric sulfate must be completely detached from the protein. This end is accomplished by a much longer treatment with barium hydroxide than indicated by Fränkel and Jellinek. If the protein is digested with the barium hydroxide solution only 3 to 4 hours, then the removal of the traces of adhering protein is a very difficult matter, as Fränkel and Jellinek found. If, however, the hydrolysis is continued for 7 hours, the removal of biuret-giving substances is accomplished without difficulty.

This circumstance is emphasized here for the reason that it indicates the existence of an intimate union between protein and polysaccharide, one more intimate than that of the ions of an ionizable salt.

The next problem to which our attention was devoted was that of the origin of the polysaccharide. Fränkel and Jellinek have not devoted much attention to this question. We prepared the polysaccharide from the material obtained on heat coagulation of egg white. One realizes now that the number of proteins, even coagulable ones, present in the egg white is very considerable. On the other hand, there is also realized the difficulty of removing all the ovomucoid from the coagulum. It was therefore considered important to compare the yields of the polysaccharide from the coagulum, from crystalline egg white, and from the ovomucoid. The increasing order of yields was as follows: (1) three times recrystallized egg white, 0.26 gm. from 100 gm.; (2) coagulum, 1.9 gm. from 100 gm.; (3) soluble ovomucoid, 5.1 gm. from 100 gm. From these observations it seems justifiable to conclude that *the source of the polysaccharide is not ovalbumin, but rather ovomucoid.*

Finally, our attention was directed to the study of the structure of the polysaccharide. The major claims of Fränkel and Jellinek regarding the composition of the substance were fully substantiated by our observations; namely, we could not detect any components other than glucosamine and mannose. With respect to the details of the structure of the substance, our findings do not fully agree with those of the earlier workers. The composition of our substance approached nearer to that of a trisaccharide or its multiple. Like the substance of Fränkel and Jellinek, it was not reducing, but the primary amino group in our substance was not substituted. Thus, the allocation of the individual sugar radicals remains unknown.

On partial hydrolysis of the non-reducing polysaccharide, a reducing trisaccharide is obtained. Whereas the original polysaccharide is precipitable from a concentrated aqueous solution by means of glacial acetic acid, the reducing trisaccharide is not precipitable by this reagent. The reducing power of the substance determined by Willstätter and Schudel's method is equal to the reducing power of one third of its weight of glucose, thus showing that the substance is a *trisaccharide.*

For the study of the further details of the structure of this trisaccharide, a larger supply of material is required than has hitherto been available.

EXPERIMENTAL.

Preparation of Polysaccharide.

From Egg White.—Egg white was coagulated in the usual way. It was hydrolyzed according to the directions of Fränkel and Jellinek by heating for 3 hours by a steam coil with 10 times its volume of 10 per cent barium hydroxide. The excess of barium was removed with carbon dioxide and after filtering, the filtrate was treated with 25 per cent neutral lead acetate until no more precipitate was formed. The precipitate contained practically none of the substances giving the α-naphthol reaction and was discarded. In some cases there was no precipitate with neutral lead acetate and, in such cases, the treatment with this reagent was omitted. The filtrate from the lead precipitate was treated with hydrogen sulfide, aerated, and filtered. The filtrate

was concentrated under reduced pressure, the temperature of the bath being kept not higher than 40°. Foaming was easily prevented by occasional addition of octyl alcohol. The light brown syrup thus obtained was taken up with a little water and the polysaccharide was precipitated by adding, alternately, barium hydroxide and a hot saturated solution of basic lead acetate. Excess of reagents was avoided as the precipitate which formed redissolved quite easily. After standing overnight in the refrigerator, the mixture was filtered with suction. The precipitate was suspended in warm water and decomposed with carbon dioxide. The mixture was filtered and the residue treated in the same way. This process was repeated four to seven times and the combined filtrates were concentrated under reduced pressure to a small volume. The barium and lead carbonate which separated on concentration were filtered off and the filtrate was treated with 10 per cent mercuric sulfate. From the filtrate, which contained the polysaccharide, mercury and sulfuric acid were removed by hydrogen sulfide and barium hydroxide respectively. The light colored solution obtained in this way was concentrated under reduced pressure and the thick syrup was poured into a large excess of dry methyl alcohol, whereupon the polysaccharide precipitated in amorphous practically colorless form. It was taken up in a little water, precipitated again with dry methyl alcohol and this process was repeated once more. The substance contained 5.5 per cent N calculated on an ash-free basis.

The product was dissolved in a minimum amount of water, poured into a large excess of glacial acetic acid, and the precipitate which formed was centrifuged off and washed several times with ether. After drying, it was dissolved in a little water and precipitated with methyl alcohol. The first sample of polysaccharide purified in this way contained 3.69 per cent nitrogen. However, in one case, it was found to contain only 2.94 per cent nitrogen. In another case, we did not succeed in obtaining nearly so low a nitrogen content either with glacial acetic acid or by any of several other methods. The details of such experiments are described in the part on egg yolk. Moreover, with glacial acetic acid the yield is not very good.

We finally succeeded in obtaining a pure substance from such material by *hydrolyzing again with barium hydroxide.*

Coagulable protein was hydrolyzed for 7 hours with 10 per cent barium hydroxide. The basic lead acetate precipitate obtained exactly as described above was dissolved in dilute acetic acid and this was centrifuged to remove a little insoluble impurity. To the clear brown filtrate a little basic lead acetate was added and it was then treated carefully with barium hydroxide until no more precipitate formed. The solution in acetic acid and precipitation by barium hydroxide were repeated at least three times. The purified lead precipitate was treated as mentioned above and the final syrup obtained after filtering off the mercury sulfide was poured into a large excess of methyl alcohol. This precipitation with methyl alcohol was repeated several times. The yield was 1.9 gm. from 100 gm. of protein.

The substance as thus prepared, and without using the glacial acetic acid method, gave no biuret reaction and had a composition corresponding to 1 molecule of glucosamine with 2 molecules of mannose. Its composition was not changed by applying the glacial acetic acid method. For instance, a product containing 3.32 per cent N was three times precipitated by glacial acetic acid and the nitrogen content was then 3.25 per cent. The polysaccharide is optically active and has the following rotations.

$$[\alpha]_D^{25} = \frac{+\ 1\ 24^\circ \times 100}{2 \times 2} = +\ 31.0^\circ \text{ in water.}$$

$$[\alpha]_D^{25} = \frac{+\ 0\ 48^\circ \times 100}{2 \times 0\ 8} = +\ 30.0^\circ \text{ in 3 per cent HCl.}$$

It has no melting point but begins to decompose at 191°. It does not reduce Fehling's solution but does so after hydrolysis by acid. It has a primary amino group. It is not precipitated by picric acid nor mercuric sulfate and only slightly by phosphotungstic acid. Its solution is neutral to litmus. It is easily soluble in water. It is insoluble in dry pyridine, alcohol, and ether.

The substances analyzed as follows:

I. 0.1018 gm. substance: 0.1590 gm. CO_2, 0.0650 gm. H_2O, and 0.0012 gm. ash.

II. 4.310 mg. substance: 6.580 mg. CO_2, 2.615 mg. H_2O, and 0.100 mg. ash.

III. 0.1176 gm. substance: 0.1856 gm. CO_2, 0.0716 gm. H_2O, and 0.0010 gm. ash.

I.	15.00 mg.	substance	: 0.40252 mg.	N	(micro-Kjeldahl).
II.	20.50 "	"	: 0.59116 "	"	"
III.	13.8 "	"	: 0.41795 "	"	"

I.	16.60 "	"	: 0.72 cc. N	(758.5 mm., 32°, Van Slyke).
III.	17.70 "	"	: 1.04 " "	(758.5 " 32°, " ").

$C_{18}H_{33}NO_{15}$.

		C	H	N	amino N	ash
Calculated.		C 42.94,	H 6.56,	N 2.78,	amino N 2.78,	ash.
Found.	I.	" 43.10,	" 7.22,	" 2.61,	" " 2.31,	" 1.17.
	II.	" 42.61,	" 6.93,	" 2.94,	" "	" 2.32.
	III.	" 43.39,	" 6.86,	" 3.05,	" " 3.12,	" 0.85.

Substances II and III were prepared by the glacial acetic acid method.

From Crystalline Egg Albumin.—Crystalline albumin was prepared in the usual way from fresh egg white and was recrystallized three times. 120 gm. of the crystalline material were boiled for 7 hours with 10 per cent barium hydroxide. The polysaccharide was isolated in the same manner as in the case of the coagulable protein of egg white and the technique was made as nearly identical as possible in order that the yields might be compared. The yield was only 0.31 gm.; *i.e.*, 0.26 gm. per 100 gm. of crystalline albumin. The substance had the same properties as described above and analyzed as follows:

5.010 mg. substance: 8.135 mg. CO_2, 2.850 mg. H_2O, and 0.110 mg. ash.
18.600 " " : 0.4393 " N (micro-Kjeldahl).
20.00 " " : 0.56 cc. N (768.7 mm., 27°, Van Slyke).

$C_{18}H_{22}NO_{15}$.

	C	H	N	amino N	ash
Calculated.	C 42.94,	H 6.56,	N 2.78,	amino N 2.78,	ash 0.
Found.	" 45.26,	" 6.49,	" 2.41,	" " 1.59,	" 2.19.

From Egg Yolk.—Fresh egg yolk was poured into acetone and the precipitate thus obtained was washed well with 95 per cent alcohol. The crude coagulable protein was heated for 3 hours with 10 per cent barium hydroxide. In this case, even after the basic lead acetate precipitate had been thoroughly purified by dissolving in acetic acid and reprecipitating with barium hydroxide, most of the preparations contained about 5 per cent nitrogen and gave a strong biuret reaction. For further purification we first tried glacial acetic acid.

5 gm. of crude polysaccharide, obtained by the above procedure and containing 5.65 per cent nitrogen, were dissolved in 5 cc. of water and this was poured into 400 cc. of glacial acetic acid. After standing for some hours, it was centrifuged and the precipitate was washed several times with ether. It was dried *in vacuo* over phosphorus pentoxide. The yield was 2.02 gm. and the nitrogen content was 4.83 per cent. It was dissolved again in 2 cc. of water, poured into 150 cc. of glacial acetic acid, and the precipitate treated as above. The yield was 0.85 gm. The substance contained 4.72 per cent nitrogen and still gave a biuret reaction.

We next tried purification with phosphotungstic acid in the presence of 5 per cent sulfuric acid. However, the fractions obtained from both the phosphotungstic acid precipitate and from the filtrate gave strong biuret reactions.

Mercuric sulfate was then tried but *no precipitate formed.*

Finally, we hydrolyzed the substance once more with barium hydroxide and succeeded in obtaining a substance entirely free from the biuret reaction. 10 gm. of the original product (N = 5.65) were boiled for 3 hours with ten times its volume of 10 per cent barium hydroxide. The precipitate obtained with basic lead acetate and barium hydroxide was purified as described above. The filtrate, freed from lead and barium, was found to be *precipitated by mercuric sulfate,* although no precipitate was formed before hydrolysis. The filtrate from the mercuric sulfate was treated in the usual way and the polysaccharide was precipitated by methyl alcohol. It contained 2.76 per cent nitrogen and gave no biuret reaction. Yield was 4 gm.

5 gm. of the same substance (N = 5.65) were again hydrolyzed and the precipitate from the basic lead acetate and barium hydroxide was not purified. After filtrating off the mercuric sulfide, the filtrate was concentrated to a thick syrup and poured into glacial acetic acid. This operation was repeated once more. The substance thus obtained contained 2.64 per cent nitrogen, while, before hydrolysis, we could not get a substance containing less than 4.72 per cent nitrogen by the same procedure. The yield was 0.9 gm.

The greater resistance of egg yolk to hydrolysis may be due to some impurity which contaminates the protein.

The pure substance had the following rotations.

$$[\alpha]_D^{25} = \frac{+0.58^\circ \times 100}{1 \times 2} = +29.0^\circ \text{ in water.}$$

$$[\alpha]_D^{25} = \frac{+0.46^\circ \times 100}{2 \times 0.8} = +28.75^\circ \text{ in 3 per cent HCl.}$$

The substance had the same properties as that from egg white and analyzed as follows:

I. 3.925 mg. substance: 6.320 mg. CO_2, 2.490 mg. H_2O, and 0.030 mg. ash.
II. 4.945 mg. substance: 7.855 mg. CO_2, 2.940 mg. H_2O, and 0.0 mg. ash.
III. 3.260 mg. substance: 5.055 mg. CO_2, 2.020 mg. H_2O, and 0.053 mg. ash.

I	21.00 mg.	substance:	0.58069 mg. N (micro-Kjeldahl).
II.	16.00 "	"	: 0.4229 " " "
III.	20.00 "	"	: 0.50688 " " "
II.	2.000 "	"	: 0.80 cc. (753 mm., 23°, Van Slyke).
III.	2.000 "	"	: 0.76 " (750.6 " 22°, " ").

$C_{18}H_{33}NO_{15}$.

		C	H	N	amino N	ash
Calculated.		C 42.94,	H 6.56,	N 2.78,	amino N 2.78,	ash 0.
Found.	I.	" 44.25,	" 7.14,	" 2.78,		" 0.76.
	II.	" 43.31,	" 6.65,	" 2.64,	" " 2.23,	" 0.
	III.	" 42.71,	" 7.00,	" 2.53,	" " 2.11,	" 1.07.

From Ovomucoid.—The preparation of the polysaccharide from ovomucoid was tried a year ago by Dr. M. L. Wolfrom in this laboratory. At that time we did not know that the conditions of hydrolysis are of the greatest importance in obtaining a substance free from protein. Crude ovomucoid was heated for 5 hours by a steam coil with 10 times its volume of 10 per cent barium hydroxide. The polysaccharide isolated from this mixture contained 8 per cent of nitrogen and the further purification was not successful with either mercuric sulfate or glacial acetic acid.

We finally prepared a polysaccharide free from biuret reaction in the following way. 100 gm. of purified ovomucoid were boiled for 7 hours with 10 times its volume of 10 per cent barium hydroxide. After removing the barium with sulfuric acid, the solution was concentrated to about 800 cc. and the polysaccharide was precipitated with barium and basic lead acetate. A test of the

solution showed that no precipitate was formed by neutral lead acetate. The lead precipitate was purified and treated as mentioned above. The yield of polysaccharide was 8.2 gm., *i.e.* 5.1 gm. from 100 gm. of mucoid. It contained 3.46 per cent of nitrogen and showed no appreciable biuret reaction. It was once more hydrolyzed for 2 hours. The substance obtained in this way analyzed as follows:

5.225 mg. substance: 8.185 mg. CO_2, 2.965 mg. H_2O, and 0.180 mg. ash.
20.00 mg. substance: 0.4873 mg. N (micro-Kjeldahl).

$C_{18}H_{33}NO_{15}$.	Calculated.	C 42.94,	H 6.56,	N 2.78,	ash 0.
	Found.	" 44.21,	" 6.57,	" 2.51,	" 3.44.

The optical rotation was not measured as the solution was quite colored.

Test for Acetyl Group.

The polysaccharides isolated from egg white, egg yolk, and ovomucoid were tested for the acetyl group by distilling with phosphoric acid or by hydrolyzing with barium hydroxide and then distilling with acid. However, we were unable to detect an acetyl group in any of the preparations.

Dialysis of Polysaccharide.

1 gm. of polysaccharide, which was prepared from egg white and which contained 3.22 per cent of nitrogen, was dissolved in 5 cc. of water and dialyzed for 17 hours at room temperature through a collodion membrane, using 80 cc. of distilled water on the outside. The solutions both inside and outside were concentrated to a small volume and precipitated by dry methyl alcohol.

The substance obtained from inside the membrane analyzed as follows:

20.000 mg. substance: 0.6006 mg. N (micro-Kjeldahl).
6.045 " " : 0.050 mg. ash.
N 3.00. Ash 0.82.

The substance from the outside analyzed as follows:

20.00 mg. substance: 0.6447 mg. N (micro-Kjeldahl).
6.790 " " : 0.040 " ash.
N 3.22. Ash 0.73.

Hydrolysis for Identification of Components.

Polysaccharide from Egg White.—2.00 gm. of polysaccharide, containing 3.36 per cent nitrogen and 7.5 per cent moisture, were heated at 100° in a sealed tube for 6 hours with 20 cc. of 20 per cent hydrochloric acid, a little norit being added for decoloration. After cooling, it was filtered and the filtrate was concentrated under reduced pressure to a small volume. During the concentration, the temperature did not exceed 35°. The residue, which contained some white crystals, was taken up with 30 cc. of absolute alcohol and allowed to stand overnight in the refrigerator. The crystals were filtered off and washed with absolute alcohol and ether. The yield was 0.5 gm. and the product analyzed correctly for glucosamine hydrochloride. It was dissolved in boiling 80 per cent alcohol and filtered, a little norit being added if necessary. To the filtrate a few drops of concentrated hydrochloric acid were added and the walls were scratched with a glass rod whereupon chitosamine hydrochloride crystallized out in beautiful thick plates. The initial rotation, 3 minutes after dissolving the product in water, was $[\alpha]_D^{25} = +96.0°$ and the equilibrium rotation was

$$[\alpha]_D^{25} = \frac{+0.72° \times 100}{1 \times 1} = +72.0° \text{ in water.}$$

The substance analyzed as follows:

20.00 mg. substance: 1.3162 mg. N (micro-Kjeldahl).
$C_6H_{14}NClO_5$ Calculated. N 6.50. Found. N 6.58.

The alcoholic filtrate from the chitosamine hydrochloride was concentrated under reduced pressure and after being neutralized, it was treated with sodium acetate and phenylhydrazine hydrochloride. A brown crystalline precipitate was thus obtained which was recrystallized from dilute alcohol. It crystallized in the form of very thin plates and is practically colorless. It was easily soluble in absolute alcohol and melted at 106°. The mixed melting point with a known specimen of levulinic acid phenylhydrazone was 107°, while the pure hydrazone melted at 107.5°. From these facts it follows that the substance is not the phenylhydrazone of mannose but of levulinic acid.

For the isolation of mannose the polysaccharide was hydrolyzed

as follows: 0.5 gm. of the substance containing 2.61 per cent of nitrogen was treated with 50 cc. of 4 per cent hydrochloric acid and heated on the steam bath for 3½ hours after adding a little norit. It was concentrated under reduced pressure to a very small volume, neutralized with sodium hydroxide and treated with sodium acetate and phenylhydrazine hydrochloride. Mannose phenylhydrazone crystallized out after scratching the walls. It was recrystallized from 50 per cent alcohol.

The optical rotation was as follows:

$[\alpha]_D^{25} = \dfrac{+0.26° \times 100}{2 \times 0.4} = +32.5°$ in equal volume mixture of water and pyridine.

A known specimen of mannose phenylhydrazone had the same rotation under exactly the same conditions, $[\alpha]_D^{25} = +32.5°$.

The substance melted at 192–193°, a mixture with pure hydrazone at 192.5–193.5°, and the pure hydrazone at 193–193.5°.

It analyzed as follows:

6 855 mg. substance: 0.621 cc. N (763 mm., 24°, micro-Dumas).
$C_{12}H_{18}N_2O_5$. Calculated. N 10.37. Found. N 10.45.

Polysaccharide from Egg Yolk.—The polysaccharide containing 2.53 per cent of nitrogen was treated in exactly the same way as in the case of egg white. The purified glucosamine hydrochloride had the following rotations: Initial rotation 3 minutes after solution was $[\alpha]_D^{25} = +97.0°$ and the equilibrium was

$$[\alpha]_D^{25} = \frac{+0.72° \times 100}{1 \times 1} = +72.0° \text{ in water.}$$

It analyzed as follows:

20.00 mg. substance: 1.2863 mg. N (micro-Kjeldahl).
$C_6H_{14}NClO_5$. Calculated. N 6.50. Found. N 6.43.

For the isolation of mannose the polysaccharide containing 3.32 per cent of nitrogen was used. The hydrazone obtained exactly as previously described had the following rotation:

$[\alpha]_D^{25} = \dfrac{+0.26° \times 100}{2 \times 0.4} = +32.5°$ in equal volume mixture of water and pyridine.

It analyzed as follows:

8.405 mg. substance: 0.739 cc. N (765 mm., 24°, micro-Dumas).
$C_{12}H_{18}N_2O_5$. Calculated. N 10.37. Found. N 10.17.

Polysaccharide from Ovomucoid.—Chitosamine hydrochloride was isolated from the polysaccharide containing 4 per cent of nitrogen and purified as usual. The optical rotation changed from an initial $[\alpha]_D^{25} = +96.77°$ to the following equilibrium value on standing.

$$[\alpha]_D^{25} = \frac{+0.72° \times 100}{1 \times 0.992} = +72.58° \text{ in water.}$$

It analyzed as follows:

20.00 mg. substance: 1.2690 mg. N (micro-Kjeldahl).
$C_6H_{14}NClO_5$. Calculated. N 6.50. Found. N 6.34.

Mannose-phenylhydrazone was also isolated in exactly the same manner as previously described. The rotation was

$$[\alpha]_D^{25} = \frac{+0.12° \times 100}{2 \times 0.2} = +30.0° \text{ in equal volume mixture of water and}$$
pyridine.

It analyzed as follows:

6.55 mg. substance: 0.577 cc. N (740 mm., 24°, micro-Dumas).
$C_{12}H_{18}N_2O_5$. Calculated. N 10.37. Found. N 9.85.

Partial Hydrolysis of Polysaccharide.

Preliminary experiments, with different concentrations of acid and times of heating, showed that the following conditions are the best for the preparation of the partially hydrolyzed substance.

3 gm. of polysaccharide, prepared from egg yolk and containing 2.53 per cent of nitrogen, were dissolved in 60 cc. of 10 per cent hydrochloric acid and hydrolyzed in a sealed tube for 20 minutes at 100°, some norit being added for decolorization. After cooling, the tube was opened and the solution was filtered. The filtrate contained 1.044 gm. of sugar, calculated as glucose, determined with Lehmann-Maquenne's method. This corresponds to 34.8 per cent of the total sugar originally present. The solution was

evaporated under reduced pressure, water being added occasionally in order to remove the excess of hydrochloric acid. It was then treated with silver carbonate and after filtering from silver chloride, was decomposed with hydrogen sulfide. The filtrate was again concentrated to a small volume and precipitated with an excess of an equal mixture of methyl and ethyl alcohols. The precipitate thus obtained was dissolved in a very little water and precipitated again with alcohol. This process was repeated several times. The product had a slight dextrorotation but it was not possible to determine it accurately owing to the color of the solution.

0.0944 gm. of the substance consumed 3.7 cc. of 0.1 N iodine solution by Willstätter and Schudel's method, while a trisaccharide would require 3.75 cc.

The substance reduces Fehling's solution. It is very soluble in water and insoluble in alcohol or ether.

It analyzed as follows:

I. 4 500 mg. substance: 6 795 mg. CO_2, 2.630 mg. H_2O, and 0.240 mg. ash.

II. 4 440 mg. substance: 6 975 mg. CO_2, 2.640 mg. H_2O, and 0.060 mg. ash.

III. 4.750 mg. substance: 8.030 mg. CO_2, 2.980 mg. H_2O, and 0.050 mg. ash.

I. 18 90 mg. substance: 0.55857 mg. N (micro-Kjeldahl).
II. 80.00 " " : 2 35 " " "
III. 20 60 " " : 0 6604 " " "
I. 23 80 " " : 0.86 cc. N (758 mm , 23°, Van Slyke).
II 20 00 " " : 1.06 " " (739 " 22°, " ").
III. 20 00 " " : 0.58 " " (739 " 23°, " ").

$C_{18}H_{33}NO_{15}$.

		C.	H	N	amino N	ash.
Calculated.		42.94.	6.56,	2.78,	2.78,	
Found.	I.	43.47,	6.89,	3.15,	2.12,	5 33.
	II.	43.41,	6.74,	4.11,	2.90,	1.35.
	III.	46.56,	7.09,	3.02,	1.58,	1.05.

ON THE MOLECULAR SIZE OF THE CARBOHYDRATES OBTAINED FROM EGG PROTEINS.

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In the preceding work of Levene and Mori[1] on the carbohydrates obtained from the egg proteins it was found impossible to prepare the substances free of mineral material without enormous losses. This fact made it impossible to determine the molecular weight of the substances by the conventional method. An approximate estimate of the molecular weight can be obtained from the diffusion coefficient of a given substance by two formulæ; one is applied to substances the molecular size of which is very large as compared with that of the solvent and the other to substances of which the molecular size is comparatively small. The first is based on the Stokes-Einstein law[2] and the second was suggested by Hüfner[3] and by Euler.[4]

The relation of the radius of the particles to the diffusion coefficient as given by Einstein is

$$D = \frac{RT}{N} \times \frac{1}{6\,\pi\,\eta\,a}$$

where D = coefficient of diffusion, R = gas constant, T = absolute temperature, N = Avogadro number, η = viscosity of the solvent, and a = radius of the particles.

The second relation is $D\,\sqrt[2]{M}$ = constant. M = molecular weight. Thovert[5] has tested the correctness of this expression on

[1] Levene, P. A., and Mori, T., *J. Biol. Chem.*, **84**, 49 (1929).

[2] Sutherland, W., *Tr. Australasian Assn. Adv. Sc.*, Jan. (1904), cited by Thovert, G., *Compt. rend. Acad.*, **150**, 270 (1910). Einstein, A., *Z. Elektrochem.*, **14**, 235 (1908).

[3] Hüfner, G., *Wied. Ann. Physik*, **60**, 134 (1897).

[4] Euler, H., *Wied. Ann. Physik*, **63**, 273 (1897).

[5] Thovert, J., *Ann. physique*, **2**, 369 (1914).

an extensive number of substances and found that for aqueous solutions at 20° the value of the constant is approximately 8×10^{-5}.

Incidentally, Thovert[6] pointed out that the two formulæ are not compatible inasmuch as according to Einstein

$$D = f \cdot \frac{1}{\sqrt[3]{M}}$$

whereas according to the other

$$D = f \frac{1}{\sqrt[2]{M}}$$

Coefficients of Diffusion in Water.

Method.—Diffusion coefficients were determined by following the diffusion through a membrane. The general procedure followed is the one described by Northrop and Anson.[7] The coefficients of diffusion were calculated according to the simplified Fick law in the form:

$$D = \frac{\Delta Q}{T} \frac{h}{\Delta C S} = \frac{\Delta Q}{T} \frac{K}{\Delta C}$$

D = diffusion coefficient in $cm.^2 \cdot sec^{-1}$
T = time in seconds.
ΔQ = amount of substance in gm. diffused in time T.
h = effective thickness of the membrane
S = effective surface of diffusion
ΔC = difference of concentration in gm. per liter on each side of the membrane. The concentration of the ouside solution being practically $= 0$, ΔC is the concentration of the inside solution.
$K = \frac{h}{S}$ constant of the cell.

Apparatus.—The capacity of the cell was 28.60 cc. The membrane used was a filter disk of Jena glass No. 4 as recommended by Northrop. The thickness of the membrane was about 0.6 mm. The cell was kept at 25° by means of a water jacket. The volume of the outside solution was 25 cc.

[6] Thovert, G., *Comp. rend. Acad.*, **150**, 270 (1910).
[7] Northrop, J. D., and Anson, M. L., *J. Gen. Physiol.*, **12**, 541 (1929).

Constant of Cell.

The following substances, saccharose, maltose, lactose, KCl, and HCl, for which the coefficient of diffusion is known to within a few per cent, were used to determine the constant of the cell.

The coefficients of diffusion for these compounds are taken from the International Critical Tables (volume 5). The values for 25° are extrapolated from values ranging from 10° to 24°.

Analyses of the amount diffused were made by optical activity measurements for lactose, saccharose, maltose and by conductivity measurements for HCl and KCl. The final volume of the samples to be analyzed was made 50 cc.

TABLE I.

Constant of the Cell.

Substances.	C Range of concentration in gm. per l. at which diffusion was measured.	$\frac{\Delta Q\ 3600}{T}$ Amount of substance diffused in gm. per hr. at 25°.	D Coefficient of diffusion in cm.^{2}·sec.$^{-1}$	K Constant of cell.
Saccharose.	99 3–99 0	0.0092_6	0.48×10^{-5}	185
	93.7–92.3	0.0087_8	0.48×10^{-5}	183
Maltose.	89.9–89.2	0.0085_0	0.46×10^{-5}	174
	87.5–89.2	0.0080_0	0.46×10^{-5}	183
Lactose.	71 5–71.1		0.46×10^{-5}	167
HCl.	3 50–3 42	0.0020_3	2.87×10^{-5}	176
	3.42–3.28	0.0019_8	2.87×10^{-5}	175
KCl.	14.7–14.3	$0\ 0056_3$	$1\ 83 \times 10^{-5}$	171
	14 3–13 8	0.0054_9	1.83×10^{-5}	170

The optical rotations were taken in a 1 meter tube with a polariscope reading to 0.001°.

The precision was such as to determine ΔQ to within ±1 per cent, the variation of C being only 0.3 per cent from an initial value of 0.290 N.

For each experiment, the amounts of substance diffused per hour were plotted against the concentration. After the diffusion of the first few per cent, the curve obtained is a straight line as required by Fick's law. The data are recorded in Table I.

Coefficient of Diffusion of Polysaccharide.

Method of Analysis of Diffused Substance.—The final volume of each sample to be analyzed was 50 cc. and the rotation was measured in a 1 meter tube.

Since the value of the specific rotation is

$$[\alpha]_D^{25} = +29.1^\circ \pm 0.2^\circ$$

TABLE II.

Progress of Diffusion of the Polysaccharide.

α_D^{25}	Time.	ΔQ	Concentration in cell.	Concentration.	Mean concentration.
degrees	*hrs.*	*gm. diffused*	*gm. per 28 6 cc.*	*per cent*	*per cent*
			1 592	100	
0 057	2.12	0.009_8	1.582	99.4	99.7
0.067	3.58	0.011_2	1.571	98 7	99.1
0 194	11.90	0.033_3	1.538	96.7	97 7
0.083	7.00	0.014_2	1.524	95.7	96 2
0 212	17.80	0.036_4	1.488	93.5	94.1
0 092	7 55	0.015_8	1.472	92.6	93 0
0 226	16.17	0.038_8	1.433	90.0	91.3
0 272	26.40	0.046_7	1.386	87.0	88.5
0 252	21.70	0.043_3	1.343	84.3	85 6
0 090	7.75	$0\ 015_5$	1.328	83.3	83.8
0 170	15 88	$0\ 029_2$	1 299	81 5	82 9

for a concentration of 0.111 gm. per 100 cc., the amount of diffused substance in gm. is given by the following expression.

$$\Delta Q \qquad 5\ 82$$

The experimental data are recorded in Table II.

The average value for ΔQ, as is best seen on a curve, is ΔQ = 0.00210 gm. per hour for a concentration of 51.0 gm. per liter. Taking the average value 175 for K we find for the coefficient of diffusion at 25°

$$D_{25^\circ} = \frac{0.00210 \times 175}{3600 \times 51} \quad 0.20 \times 10^{-5}$$

Coefficient of Diffusion of Product of Hydrolysis of the Polysaccharide.

Method of Analysis of Diffused Substance.—The amount of carbohydrate diffused was determined by applying the method of Willstätter and Schudel.[8] The conditions were modified slightly on account of the dilution of the solutions to be titrated. 5 cc. of 0.1 N NaOH and 10 cc. of 0.02 N iodine were added to the sample to be analyzed. The final volume was made up to 50 cc. 60 minutes later the solution was acidified and titrated with 0.02 N $Na_2S_2O_3$.

As, under these conditions, the amounts of iodine used were not exactly proportional to the amounts of sugar, a calibration curve was prepared by plotting the relative amounts of iodine and sugar. The experimental data are recorded in Table III.

TABLE III.

*Progress of Diffusion of the Trisaccharide.**

0 02 N iodine used.	Time.	Gm. diffused per hr.	Average concentration in cell. 100 per cent = 20.1 gm. per l.
cc.	*hrs.*		
3.33	6.26	0.0022_7	98.81
7.38	16.33	0.0020_0	94.8
2.46	5.42	0.0017_1	91.1
2 94	7.77	0.0015_4	89.2
3 55	11.03	0.0014_0	87.0
2.90	7.35	0.0015_9	84.5
6.62	22.55	0.0012_5	81.1
4.54	18.15	0.0011_6	76.8

* Concentration of the starting solution: 2.01 per cent.

The decrease of the amount of diffused substance per unit of time against concentration was too large to be explained by Fick's law. It could be concluded that the substance was not pure. The average coefficient of diffusion for 25° lies between the following values.

$$D_{25^\circ} = \frac{0.00124 \times 175}{3600 \times 16.3} = 0.37 \times 10^{-5}$$

$$D_{25^\circ} = \frac{0.00140 \times 175}{3600 \times 17.6} = 0.38_5 \times 10^{-5}$$

[8] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, **51**, 780 (1918).

Approximate Molecular Weights of the Two Carbohydrates.

These molecular weights were calculated by the relation

$$D \sqrt[2]{M} = \text{constant} = 8 \times 10^{-5} \text{ at } 20°.$$

The diffusion coefficients determined at 25° were reduced to 20° with an average temperature coefficient.

Polysaccharide.

For this substance

$$D_{25°} = 0.20 \times 10^{-5}$$

$$D_{20°} = 0.18 \times 10^{-5}$$

Hence

$$0.18 \times 10^{-5} \sqrt[2]{M} = 8 \times 10^{-5}$$

from which it follows that the molecular weight is approximately 2000.

Product of Hydrolysis of Polysaccharide.

For this substance

$$D_{25°} = 0.37 \times 10^{-5} \text{ to } 0.38_5 \times 10^{-5}$$

$$D_{20°} = 0.35 \times 10^{-5} \text{ (approximately)}$$

Hence

$$0.35 \times 10^{-5} \sqrt[2]{M} = 8 \times 10^{-5}$$

from which it follows that the molecular weight is approximately 500.

It may be added that the diffusion coefficient for crystalline raffinose is 0.36×10^{-5} at 20°.

CONCLUSION.

On the basis of the diffusion coefficient the polysaccharide prepared from the egg proteins consists of four trisaccharides, each having an approximate molecular weight of 500, which weight corresponds to that required by theory for the substance consisting of 1 molecule of glucosamine and of 2 molecules of mannose.

A MICRO TIME METHOD FOR DETERMINATION OF REDUCING SUGARS, AND ITS APPLICATION TO ANALYSIS OF BLOOD AND URINE.

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The author and Van Slyke (5) have recently published a method for sugar determination in which the time required to decolorize a standard ferricyanide solution was used as a measure of the sugar content. The convenience which this method demonstrated in clinical work stimulated attempts to widen its range of usefulness by developing a micro modification. By carrying out the reaction in small test-tubes heated in a porcelain casserole, which provides a brilliant white background, it has proved possible without decrease in accuracy to make duplicate determinations on a 0.2 cc. sample of blood.

We regret that at the first publication of the time method (5) we overlooked the fact that Cole (1) had previously employed for urine analysis the principle of measuring sugar content by the time required for reducing a colored reagent.[1] The latter was a copper solution, and has apparently not been adapted to the small amounts of sugar encountered in blood.

DETERMINATION OF REDUCING SUGAR IN BLOOD.

Reagents.

Potassium Ferricyanide Reagent.—This reagent is the same used by Hawkins and Van Slyke (5), except that the present solution

[1] Although apparently not published until 1926, the method was reported by Professor Cole to the Biochemical Society in 1922.

contains only 0.5 gm. of potassium ferricyanide, instead of 1.0 gm. per liter.[2]

Tungstic Acid Solution (Mixed Reagents of Folin and Wu (3)). —The solution is the same as that previously used by Van Slyke and Hawkins (6) except that 1 volume of 10 per cent sodium tungstate and 1 volume of $\frac{2}{3}$ N sulfuric acid are mixed with 8 volumes of distilled water.

Procedure for Blood Sugar Determination.

Precipitation of Blood Proteins.—When samples of capillary blood are to be analyzed, a sufficient number of rubber-stoppered tubes (15 × 100 mm.) are prepared each containing exactly 2 cc. of the tungstic acid precipitating reagent described above. The blood drops forming on the incised finger or ear lobe are drawn by capillary attraction into a 0.200 cc. capillary pipette, which is at once emptied into one of the test-tubes. The pipette is rinsed twice by drawing the tungstic acid up into it. The test-tube is then stoppered and shaken. The blood is diluted 1:11 by this procedure. After 2 minutes the mixture is filtered through a dry filter paper (4.5 cm.) into a test-tube (15 × 100 mm.).

If the blood is known to be hyperglycemic, a portion of the filtrate is diluted with an equal volume of water.

When blood analyses are to be made which require a Folin-Wu filtrate where the blood is diluted 1:10, this filtrate can be used instead of the 1:11 blood filtrate.

The 0.200 cc. pipette is made from a capillary tube of about 1 mm. bore, and is calibrated by weighing 2.71 gm. of mercury in the dry pipette.

Decolorization of Ferricyanide by Blood Filtrate.—0.5 cc. of filtrate is pipetted into a Pyrex test-tube, of 9 × 90 mm. outside measure[3] followed by 0.5 cc. of ferricyanide solution. Both

[2] The potassium ferricyanide should not contain potassium ferrocyanide. The potassium ferricyanide may be tested as follows. To 10 cc. of 0.5 per cent potassium ferricyanide add 0.5 cc. of a 0.5 per cent solution of ferric chloride and 1 drop of N HCl. If ferrocyanide is present in quantity sufficient to be 0.1 per cent of the ferricyanide, under these conditions, a green color appears on the addition of the ferric chloride. C.P. ferricyanide reagents we have tested did not develop any green color.

[3] The tubes were made to order for us from standard thin wall Pyrex glass tubing, internal diameter 8 mm. The thickness of the glass wall is 0.6 mm.

solutions must be measured accurately from Ostwald bulb pipettes with capillary stems. The ferricyanide should be added last, in order to facilitate its mixture with the lighter filtrate. The tube is shaken to mix the two solutions and is then immersed in water in a white glazed casserole in which the water is boiling gently. The casserole has a diameter of 95 mm. so that the tubes rest with their mouths on the edge, but cannot slip into the water. Two or three tubes may be heated at once. A tube containing distilled water is immersed with the other tubes to facilitate by comparison the detection of the moment when the ferricyanide solutions are decolorized. The time in seconds for each tube is taken, preferably with a stop-watch, from the moment the tube is immersed in the boiling water until the last trace of yellow disappears.

With the prescribed amount of ferricyanide and size of test-tubes, 50 mg. of sugar per 100 cc. of blood decolorize the reagent in 520 seconds, and 300 mg. of glucose per 100 cc. of blood will decolorize it in 76 seconds, when the blood filtrate represents a 1:11 dilution of the blood. If blood filtrate represents a 1:10 dilution for blood, the decolorization times are correspondingly shorter, as shown by Fig. 1. It is possible to work to the above time limits. However, if the reagent is decolorized in less than 110 seconds (equivalent to more than 150 mg. per cent of blood sugar for blood diluted 1:11, and to more than 140 mg. per cent of blood sugar for blood diluted 1:10) it is best to dilute another portion of blood filtrate with an equal volume of water and to repeat the analysis with the diluted filtrate in order to obtain a longer decolorizing period and more exact results.

Graphic Calculation of Results of Blood Analysis.

The number of mg. of sugar per 100 cc. of blood is read directly from the appropriate curves shown in Fig. 1 when the 0.5 cc. of filtrate used represents a 1:11 or a 1:10 dilution of the blood. When the filtrate is twice as dilute (hyperglycemic blood) the blood sugar content indicated by the appropriate curve is doubled.

DETERMINATION OF REDUCING SUGAR IN URINE.

The procedure outlined below is designed for use with the same apparatus and reagents with urine that are employed in the above

micro blood analysis. Such a micro method is not required for urine, but we describe it for the reason that it makes available for urine the same technique and apparatus used for blood. The urine analysis is planned for use with urines, such as those encountered in diabetes, in which the glucosuria is so gross that its significant variations can be satisfactorily shown by measurement of the total reducing substances. The method is accurate to within 0.1 gm. of glucose per 100 cc. of urine. Unusually concentrated normal urines may have reducing substances equivalent in reducing power to as much as 0.4 per cent glucose. The normal reducing substances, however, have been found by previous authors (2, 4, 7) to be almost entirely non-fermentable, and to have no apparent relationship to carbohydrate metabolism. Accordingly the technique is regulated to determine reducing substances in concentrated urine in concentrations of 0.5 per cent and above, and in dilute urine in concentrations of 0.25 per cent and above.

Reagents for Urine Sugar.

Ferricyanide Solution.—Same as for blood sugar.

Procedure for Urine Sugar.

Dilution of Urine.—Ordinarily 1 cc. of urine is diluted with water to 100 cc. In urine so diluted the ferricyanide reagent will determine up to 3 per cent of glucose. In case the sugar content is known to be above 2 per cent, 1 cc. of urine is diluted to 200 or 400 cc., so that glucose up to 6 and 12 per cent respectively can be determined. On the other hand, if the urine as voided is presumably of low sugar content, it is best to dilute only 25-fold. Dilution is the only preliminary treatment of the urine required, as even albumin does not affect the determination.

Decolorization of Ferricyanide by Diluted Urine.—0.5 cc. of diluted urine is pipetted into a Pyrex test-tube (9 × 90 mm. outside measurement) followed by 0.5 cc. of ferricyanide solution. The procedure from this point is exactly the same as in the blood sugar method described above.

The amount of sugar in the urine is found by use of the curve in Fig. 1. If the urine has been diluted 200-fold instead of 100-fold, the sugar concentrations indicated by the curve are doubled.

On the other hand, they are halved if the dilution has been only 50-fold.

EXPERIMENTAL.

In order to determine the rate at which glucose reduces ferricyanide, the reagent was heated with standard glucose solutions of

TABLE I.

Determination of Time Required by Various Concentrations of Glucose to Decolorize Ferricyanide Reagent under Conditions of Micro Blood Determination, When Blood is Diluted 1:11.

Glucose concentration in standard solutions, *mg. per cc.*	0.2727	0.1818	0.1364	0.0909	0.0682	0.0455
Glucose represented in portion of solution used for determination, *mg.*	0.1364	0.0909	0.0682	0.0455	0.0341	0.0228
Time required to remove color from reagent, *sec.*	75	98	126	184	269	510
	76	98	120	183	269	520
	77	98	123	185	270	520
Average	76	98	123	184	269	520

TABLE II.

Determination of Time Required by Various Concentrations of Glucose to Decolorize Ferricyanide Reagent under Conditions of Blood Determination, When Blood is Diluted 1:10.

Glucose concentration in standard solutions, *mg. per cc.*	0.3	0.2	0.15	0.10	0.075	0.05
Glucose represented in portion of solution used for determination, *mg.*	0.15	0.1	0.075	0.05	0.0375	0.025
Time required to remove color from reagent, *sec.*	70	91	114	164	240	449
	71	91	115	168	236	449
	72	91	113	166	238	455
Average	71	91	114	166	238	451

various concentrations covering the ranges encountered in blood and urine analyses. 1 volume of reagent was mixed with 1 volume of glucose solution. The results are shown in Fig. 1, and Tables I and II. Table I gives the results obtained under the conditions

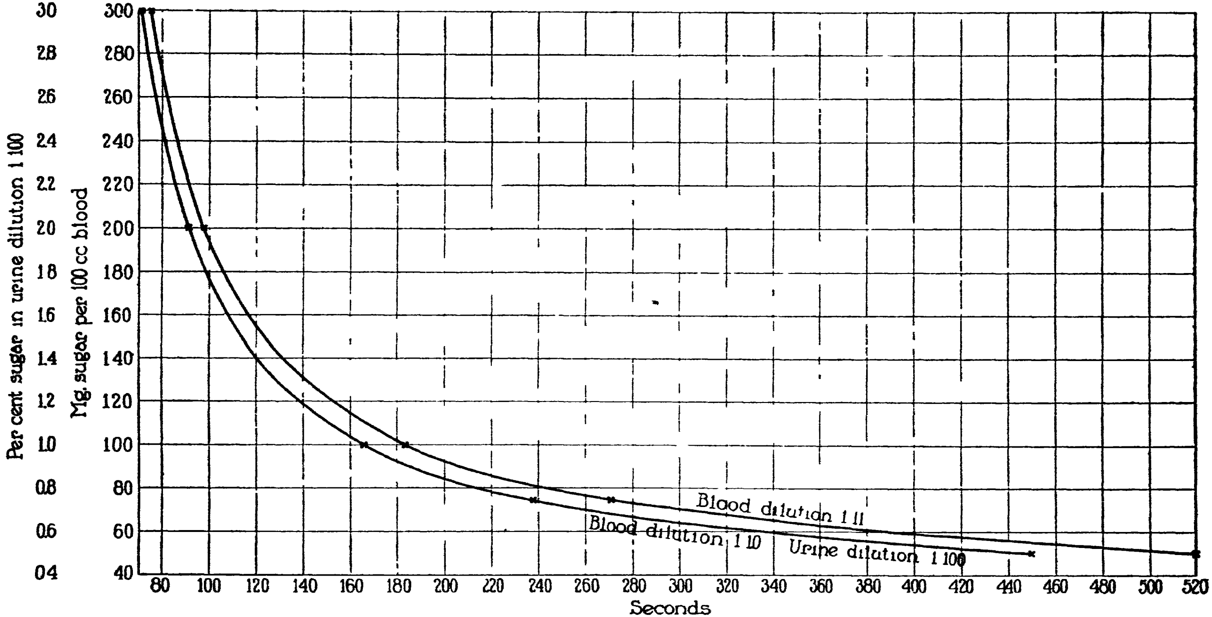

Fig. 1. Time curves of reduction of ferricyanide by standard glucose solutions under conditions of blood and urine determinations. Abscissæ represent time in seconds required by glucose standards to decolorize ferricyanide reagent. Each cross represents the average of several determinations with glucose standards. Ordinates by use of the appropriate curve represent per cent of sugar in urine when urine is diluted 1:100, or mg. of sugar per 100 cc. of blood when blood is diluted 1:10 or 1:11. If other dilutions are used, sugar contents represented by the ordinates are multiplied or divided accordingly. *E.g*, if urine dilution is 1:50 instead of 1:100, multiply by 0.5 the sugar content indicated by the curve.

described for the micro blood method, where 0.2 cc. of blood is added to 2.0 cc. of tungstic acid reagent. Table II gives the results obtained under the conditions described for the urine method, and for the blood method with filtrate representing a 10-

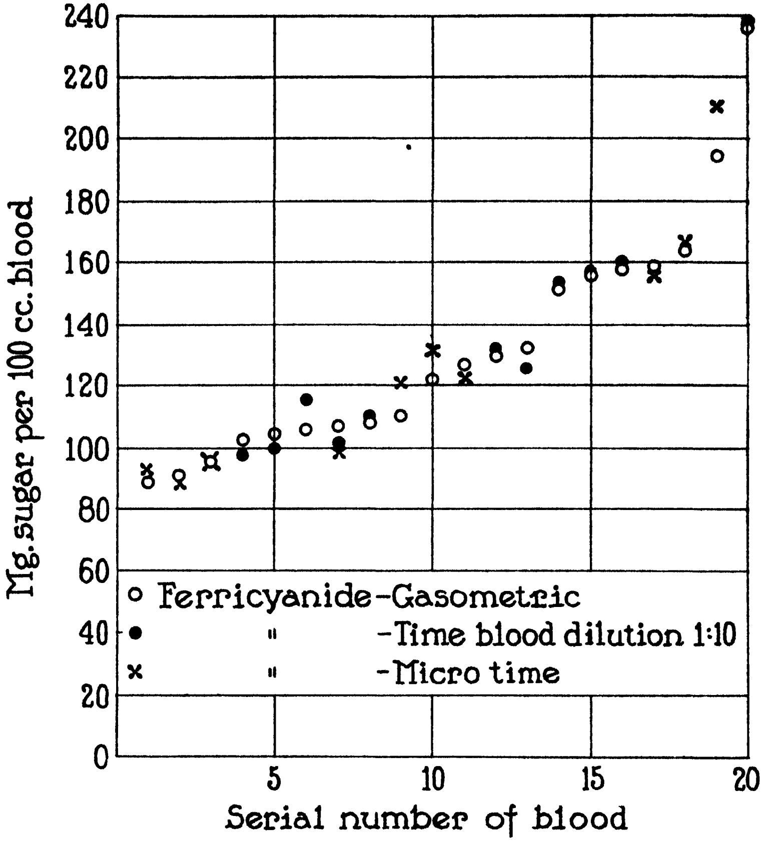

Fig. 2. Comparison of sugar found in blood by the Van Slyke-Hawkins gasometric and the time methods. Ordinates represent mg. of sugar per 100 cc. of blood. Each symbol represents the average of duplicate determinations.

fold dilution of blood. The results indicate that a reproducible curve is obtained by means of which it is possible to determine the amount of glucose in a solution from the time taken to decolorize the ferricyanide reagent.

Comparison with Gasometric Blood Sugar Method.

Comparison of results obtained by the Van Slyke-Hawkins gasometric method with those yielded by the present blood sugar method in analyses of twenty bloods, normal and pathological, is shown in Fig. 2. The two methods agreed, usually within a few mg. per 100 cc. In these analyses the dilutions of blood filtrate, either 1 volume of blood and 10 volumes of precipitant, or 1 volume of blood and 9 volumes of precipitant were used.

TABLE III.

Comparison of Van Slyke-Hawkins Gasometric and Micro Time Methods for Sugar in Diabetic Urine.

Diabetic urine No.	Per cent sugar.	
	Gasometric.	Time.
1	2.89	2.80
2	2.42	2.46
3	1.69	1.68
4	1.57	1.39
5	1.25	1.20
6	1.00	0.88
7	0.38	0.33
8	0.35	0.33

Comparison with Gasometric Urine Sugar Method.

In Table III are given the results of analyses of diabetic urines and the comparison with the Van Slyke-Hawkins method. The two methods agree within 0.1 gm. of sugar per 100 cc. of urine.

SUMMARY.

The method of Hawkins and Van Slyke (5), in which sugars are estimated from the time they require to reduce a known amount of yellow ferricyanide solution completely to colorless ferrocyanide, has been refined so that 0.2 cc. of blood suffices for duplicate analyses.

The accuracy, ± 5 per cent of the amount determined, and the time required (75 to 300 seconds) are the same as with larger samples and the previous technique.

Use for urine analyses of the same reagents and apparatus employed for blood sugar determination is described.

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REDUCING POWERS OF DIFFERENT SUGARS FOR THE FERRICYANIDE REAGENT USED IN THE GASOMETRIC SUGAR METHOD.

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In the present paper the reducing values of some commonly used sugars are compared with those of glucose under the conditions of the Van Slyke-Hawkins (1928) gasometric method for sugar determination. This method depends upon the quantitative reduction of potassium ferricyanide in an alkaline solution powerfully buffered by a mixture of K_2CO_3 and $KHCO_3$, the excess ferric salt being afterwards measured by the amount of nitrogen gas which it frees from an excess of hydrazine in the Van Slyke-Neill (1924) manometric apparatus. The comparative reducing values of different sugars under the conditions of several methods of determination have been measured by Bertrand (1906), Greenwald, Samet, and Gross (1924–25), Pucher and Finch (1926), and others. Their data show that the relative reducing values of different sugars vary greatly with the conditions of analysis. Consequently when a new method of sugar determination is introduced the reduction factors must be ascertained for each individual sugar before the latter can be determined by the method.

EXPERIMENTAL.

Reduction of Ferricyanide by Various Sugars.

In order to determine the extent to which the various sugars reduce the ferricyanide reagent, containing 8 gm. of potassium ferricyanide and 75 gm. each of potassium carbonate and bicarbonate, the reagent was heated with standard sugar solutions

TABLE I.

Glucose Equivalents of Various Sugars Determined by Dividing N_2 Pressure Fall per Mg. of Sugar by N_2 Pressure Fall per Mg. of Glucose and Their Comparison to Those Found by Bertrand.

Sugar.	Concentration of sugar in standard solution.	Sugar represented in portion of solution used for gasometric determinations.	N_2 pressure fall per mg. sugar at 25°.	Glucose equivalent.	
				N_2 fall per mg. sugar divided by N_2 fall per mg. glucose.	Mg. Cu reduced per 10 mg. sugar divided by mg. Cu reduced per 10 mg. glucose (Bertrand).
	mg. per cc.	*mg.*	*mm.*		
Glucose.	0.2	0.4	349.5	1.0	
	0.1	0.2	349.5	1.0	1.0
	0.05	0.1	349.5	1.0	
Mannose.	0.2	0.4	353	1.01	
	0.1	0.2	354	1.013	1.0
	0.05	0.1	356	1.018	
Galactose.	0.2	0.4	270	0.772	
	0.1	0.2	276	0.790	0.94
	0.05	0.1	277	0.794	
Fructose.	0.2	0.4	329	0.942	
	0.1	0.2	343	0.984	
	0.05	0.1	346	0.990	
Arabinose.	0.2	0.4	321	0.916	
	0.1	0.2	330	0.944	1.04
	0.05	0.1	333	0.954	
Xylose.	0.2	0.4	339	0.960	
	0.1	0.2	356	1.017	1.00
	0.05	0.1	357	1.020	
Maltose.	0.2	0.4	251	0.718	
	0.1	0.2	253	0.724	0.55
	0.05	0.1	256	0.732	
Lactose.	0.2	0.4	253	0.724	
	0.1	0.2	252	0.721	0.705
	0.05	0.1	256	0.732	

and the determinations carried out under the conditions described for macro blood analyses (Van Slyke and Hawkins, 1928). 1.5 cc. of the ferricyanide reagent were mixed with 3.0 cc. of 0.02, 0.01, and 0.005 per cent solutions of the various sugars in the usual test-tubes and were heated for 20 minutes.[1] Controls for the ferricyanide sugar solutions made by mixing 1 volume of ferricyanide reagent with 2 volumes of distilled water, were run at the same time to determine p_0 readings.

The results are shown in Table I. They indicate that the amount of ferricyanide reduced is directly proportional to the amount of glucose, mannose, maltose, or lactose present. The amount of ferricyanide reduced is directly proportional to the amount of fructose, arabinose, or xylose present when the concentration of these sugars does not exceed 0.1 mg. per cc. in the standard solution. The difference in amount of ferricyanide reduced per mg. of sugar when the concentration of fructose, arabinose, or xylose is 0.2 mg. per cc. in the standard solution is only 5 per cent less than when the concentration is 0.1 mg. or less in the standard solution. The relative reducing powers of mannose, xylose, and lactose, compared with glucose, are the same for the ferricyanide reagent as those found by Bertrand for his copper reagent.

Determination of Reducing Sugar in Solution.

It is possible to determine the concentrations of these sugars when one sugar alone is present in solution by use of the factors and conditions described for the macro blood gasometric method. The sugar content calculated by the glucose factors of Van Slyke and Hawkins is divided by the factor in the next to the last column of Table I to give mg. of the sugar determined per 100 cc. of solution.

SUMMARY.

Glucose reducing equivalents of mannose, galactose, fructose, arabinose, xylose, maltose, and lactose for potassium ferricyanide have been determined under conditions of the macro blood gasometric method of Van Slyke and Hawkins.

[1] The anhydrous sugars used were kindly given to me by Dr. P. A. Levene.

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STUDIES ON BLOOD CELL METABOLISM.

IV. THE EFFECT OF METHYLENE BLUE UPON THE OXYGEN CONSUMPTION, GLYCOLYSIS, AND LACTIC ACID FORMATION IN LEUCOCYTES.

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(Received for publication, June 28, 1929.)

It has previously been shown that methylene blue and other reversible dyes have a marked effect on the oxygen consumption and glycolysis of erythrocytes and on the oxygen consumption of sea urchin and starfish eggs (1). The increase of oxygen consumption due to methylene blue was found to be proportional to the rate of anaerobic glycolysis, enormously increased in mammalian erythrocytes where the respiration is almost nil, and much less marked in avian nucleated erythrocytes. It was of interest to study the action of methylene blue upon the respiration and glycolysis of cells possessing a high aerobic metabolism; namely, leucocytes. These leucocytes were obtained from three different sources: human blood, dog lymph, and rabbit peritoneal exudates.

The oxygen consumption of normal and abnormal leucocytes was in general increased after addition of methylene blue but the increase was less than the corresponding effect on erythrocytes. Very rarely no increase was observed. As an example of the difference in the action of methylene blue on erythrocytes and leucocytes we give the following experiment which has been repeated consistently several times. Heparinized human blood was centrifugalized for 2 minutes; the supernatant plasma, containing the leucocytes and a few red cells, was separated from the bottom layer containing the erythrocytes, and the oxygen consumption of both kinds of cells was studied at 37.5° in Warburg's vessels with Barcroft manometers, before and after addition

of methylene blue. While in the case of leucocytes the increase produced by the addition of methylene blue was 38 per cent, in the case of erythrocytes this increase reached 46.3 per cent (Chart 1). There is no distinct difference in the action of methylene blue on granulocytic and non-granulocytic cells (Table I).

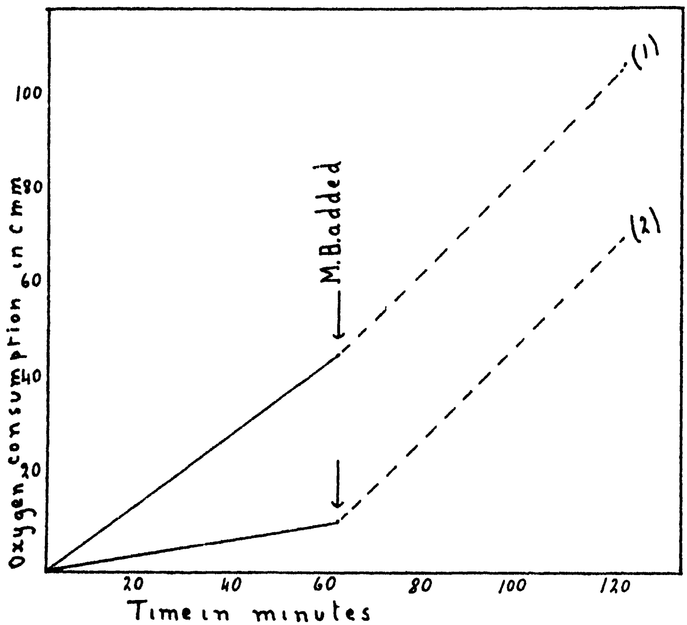

CHART 1 The effect of methylene blue on the oxygen consumption of leucocytes and erythrocytes. Human blood, myelogenous leucemia. After short centrifugation, the supernatant plasma containing leucocytes is separated from the red cells. Curve 1, effect of methylene blue on leucocytes; Curve 2, effect of methylene blue on erythrocytes.

As has been found in the case of erythrocytes and marine eggs, the addition of KCN in concentration sufficient to inhibit the respiration of leucocytes did not affect the action of methylene blue. This action was, indeed, more marked compared with the corresponding action on leucocytes alone (Table II). The increased anaerobic glycolysis produced by KCN undoubtedly accounts for this fact. This relationship between anaerobic

glycolysis and the oxidative effect of methylene blue holds only within certain limits; *i.e.*, methylene blue exerts its action even on cells with high aerobic metabolism, although in lesser degree.

Recently Wieland and Bertho (2) investigating the mechanism of the fermentation of alcohol to acetic acid by *Bacterium orleaneuses*, have found that when benzoquinone is used as a hydrogen

TABLE I.

Effect of Methylene Blue (0.005 Per Cent) upon Oxygen Consumption of Leucocytes.

Type of cells.	O_2 consumption.		Increase due to methylene blue.
	Control.	After addition of methylene blue (0.005 per cent).	
	c.mm. per hr.	*c.mm. per hr.*	*per cent*
Lymphocytes.			
Dog lymph.	38.0	60	79
	23.1	33.8	60
	21.8	19.1	No increase.
Human blood.			
Lymphatic leucemia.	55.9	68.5	22.6
	50.0	65.7	31.4
	52.2	60	15.1
Polynuclear leucocytes.			
Human blood.			
Myelogenous leucemia.	34.2	54	58
	45.0	61.6	37
	60.0	100	66
	118.0	163	38
	127.0	169	24.8
	98.4	75	No increase.
	268.0	362	26
Empyema.	31.4	26.7	No increase.
Polycythemia.	60.0	70	16.6

acceptor in the absence of free oxygen, the dehydrogenation is more rapid. With methylene blue as hydrogen acceptor the reaction was very slow. They explain this discrepancy by assuming that methylene blue does not penetrate the walls of the cell and reacts only on its outer surface. Penetration of methylene blue (or some of the less methylized dyes always present in the purest samples of the dye) through the cell membrane was sug-

gested as an explanation for the latent period observed in sea urchin and starfish eggs, before the increased oxygen consumption due to addition of dye could be manifested. If the oxidative

TABLE II.

Effect of KCN on Oxygen Consumption of Leucocytes.

Human blood leucocytes.	O_2 consumption.		Increase after addition of methylene blue.
	Control, 0.002 M KCN added	After addition of methylene blue.	
	c mm. per hr	*c mm. per hr.*	*per cent*
Myelogenous leucemia.	41	86	110
	27	45	67
	11	73	551
	37	120	219
Empyema.	2	15.6	680
Polycythemia.	14	54	286
Lymphatic leucemia.	14	32	128

TABLE III.

Effect of Methylene Blue upon Glycolysis of Leucocytes.

Rabbit leucocytes from peritoneal exudate after Hamburger's method. Centrifuged and kept in Ringer-Tyrode solution pH 7.4. Glucose content 0.150 per cent. Incubation period 3 hours at 37.5°.

	Glucose.			Lactic acid.			Oxidation by methylene blue calculated as glucose.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.	
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
Control................	5.78	3.75	2.03	0.57	5.36	4.79	
Methylene blue added.		3.62	2.16		5.05	4.48	0.60
Control................	6.06	3.09	2.97	0.71	5.90	4.19	
Methylene blue added..		4.19	1.87		2.95	2.24	0.40
Control................	6.28	3.95	2.33	0.82	5.44	4.62	
Methylene blue added..		3.33	2.95		5.68	4.86	0.99
Control................	6.15	4.45	1.70	0.93	4.05	3.12	
Methylene blue added..		4.50	1.65		3.25	2.32	0.70

action of methylene blue is exerted only through the carbohydrate oxidation, evidently we can dispense with the necessity for diffusion of the dye into the cell. There is much evidence in favor of considering the glycolytic process as one which occurs at the cell

interface. Since methylene blue is strongly adsorbed by the cell surface it can play its rôle of hydrogen acceptor, even where free oxygen is available for this process.

The action of methylene blue on the glycolysis and lactic acid formation of leucocytes is also less marked than the corresponding effect on mammalian erythrocytes. Thus while the oxidative action of methylene blue on the carbohydrate metabolism of erythrocytes (calculated as mM per liter of sugar oxidized) goes from 1.14 to 2.55, the amount of glucose oxidized on leucocytes is only from 0.40 to 0.99 (Table III).

CONCLUSIONS.

Methylene blue increases the oxygen consumption of leucocytes. It also increases the oxidation of glucose during glycolysis as manifested by a diminished lactic acid formation.

KCN has no influence on these processes.

There is no appreciable difference of the methylene blue action between granulocytic and non-granulocytic leucocytes.

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STUDIES ON BLOOD CELL METABOLISM.

V. THE METABOLISM OF LEUCOCYTES.

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In previous papers (1) we have reported studies on the oxygen consumption and on the lactic acid production, under aerobic and anaerobic conditions, in various types of erythrocytes and other cells, and on the effect of methylene blue and other dyes on these processes. In the present paper we are concerned with the metabolic behavior of the leucocytes of the blood, with a comparison of the cells of the granulocytic series with the non-granulocytic elements, and, so far as possible, with cells of different stages of maturity. It was finally desired to determine in how far the metabolic behavior of the white cells of leucemic blood corresponds to that of cancer cells or to that of the cells of embryonic tissues.

It is at once evident that the colorless elements of the blood, cell for cell, have a much higher metabolic activity than do the erythrocytes. The increased rate at which leucemic blood (or blood in which a high degree of leucocytosis exists) darkens on standing, indicative of inceased oxygen utilization, is a matter of every day clinical observation.

Although the sugar-splitting power of the leucocytes was known for a long time, the production of lactic acid was clearly demonstrated only through Levene and Meyer's (2) studies. This qualitative demonstration was followed later by Slosse's (3) quantitative determinations. Maclean and Weir (4) conclude that leucocytes have from 200 to 1000 times the glycolytic activity of erythrocytes. The earlier work is considered in a recent review by Fleischmann (5). The metabolism of the polynuclear and the mononuclear leucocytes obtained from sterile

exudates has been studied by Bakker (6), who used the material formed after injections of various materials (Hamburger (7)) into the peritoneal cavity of rabbits. He concluded that both polynuclear leucocytes and mononuclear cells behave in their metabolism like cancer cells, inasmuch as they have a high anaerobic metabolism, while the splitting of sugar into lactic acid is not materially reduced under aerobic conditions Fleischmann and Kubowitz (8), using goose and rabbit leucocytes suspended in Ringer's solution, concluded that the oxygen consumption was much higher (10 times greater) than Bakker had found it and that the fermentation also was appreciably greater. Fujita (9), in Warburg's laboratory, studied leucocytes from rat blood which he received into citrate solution. The leucocytes were then removed from the upper layer after brief centrifugalization. When his experiments were prolonged, the oxygen consumption was greatly reduced, but in short experiments in which the oxygen utilization was not damaged, he found that leucocytes have a metabolism similar, not to cancer tissue, but to normal embryonic cells; that is, a large anaerobic glycolysis, which under aerobic conditions, is nearly replaced by the respiration. In Warburg's nomenclature,[1] the average results of his series were as follows·

$$Q_{O_2} = -9, \; Q_M^{O_2} = 1\,9, \; Q_M^{N_2} = 19\,9$$

It is evidently of prime importance at the outset to determine the effects of external agents upon the metabolism of leucocytes. Of the two fundamental sources of cell energy, respiration and fermentation, the first is far more sensitive to external injuries than the second. While the fermentative process goes on as long as the cell surface and the glycolytic enzyme remain unaltered, the respiratory process is readily injured by the action of external agents that do not affect the cell surface or the respiratory ferment, if the correctness of Warburg's hypothesis is assumed. Early in the course of this study, in an effort to obtain a constant source of "normal" material, we decided to employ peritoneal exudates from the rabbit, following closely Hamburger's (7) methods. The results obtained were extremely irregular. In some cases the cells would respire, reduce methylene blue, and cause glycolysis both aerobically and anaerobically, but in other instances their respiratory power was lost, although glycolysis and the power to reduce methylene blue remained intact. Evidence of this loss of

[1] Q_{O_2} represents the amount of oxygen consumed per unit of material and per unit of time, $Q_M^{O_2}$ the amount of lactic acid under aerobic, and $Q_M^{N_2}$ the amount of lactic acid under anaerobic conditions, per unit of material and per unit of time.

oxidative power was also proved by the high glycolytic quotient in aerobic glycolysis, which in some cases reached almost as high a value as during anaerobic glycolysis (Table I). We then found

TABLE I.

Glycolysis of Rabbit Leucocytes.

Values for sugar and lactic acid are expressed in mM per liter.

	Sugar.			Lactic acid.			Glycolytic quotient.	Method of producing anaerobiosis.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.		
Aerobic.								
Polynuclear leucocytes.								
I	5.69	2.04	3.65	0.74	7.94	7.20	0.98	
II	6.06	3.09	2.97	0.71	5.90	4.19	0.70	
III	6.34	4.55	1.79	0.45	2.50	2.05	0.57	
IV	6.28	3.33	2.95	0.82	5.68	4.86	0.82	
V	6.15	4.50	1.65	0.93	3.25	2.32	0.70	
Lymphocytes.								
Sample I	4.73	4.15	0.58	0.74	1.80	1.06	0.92	
Anaerobic.								
Polynuclear leucocytes.								
I.	5.69	2.72	2.97	0.74	7.38	6.64	1.11	Nitrogen atmosphere.
II.	6.06	2.35	3.71	0.71	7.61	6.90	0.93	Nitrogen atmosphere.
III.	6.34	4.86	1.48	0.45	2.79	2.34	0.79	Nitrogen atmosphere.
IV	6.28	3.83	2.45	0.82	6.15	5.33	1.08	0.002 M KCN.
V.	6.15	4.39	1.76	0.93	4.19	3.26	0.93	0.002 " "
Lymphocytes.								
Sample I	4.73	4.08	0.65	0.74	2.03	1.29	1.00	Nitrogen atmosphere.

that centrifugation caused this damage to the respiration and that when sodium citrate was employed to avoid clotting, according to Hamburger's technique, cell metabolism was further impaired. We therefore abandoned this method. Shortly afterward Fujita's

paper appeared confirming our observation upon the damage done by centrifugalization and showing also that change of temperature similarly has a harmful effect on respiration.

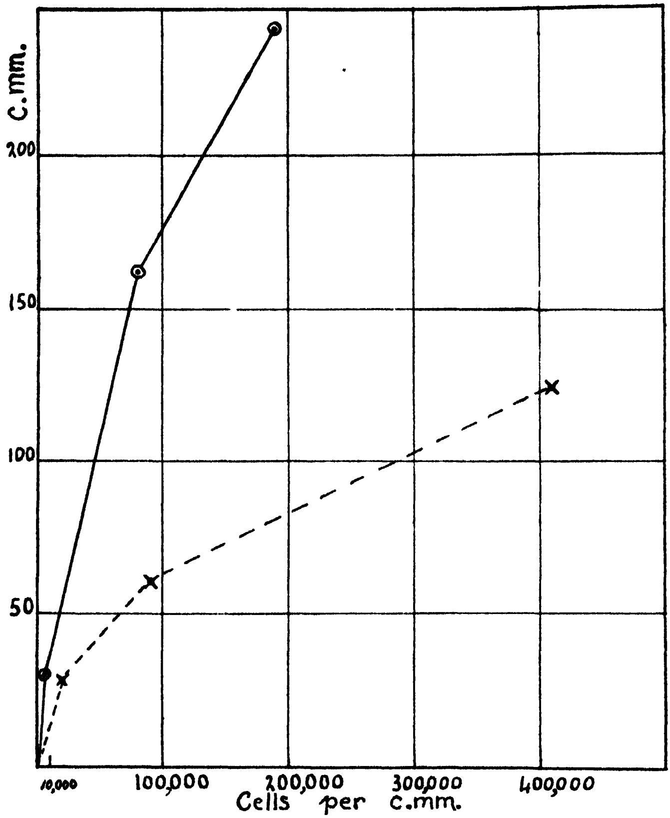

CHART 1. Variation of the oxygen consumption of leucocytes with variation in their concentration. The solid line represents oxygen consumption of granulocytes (material from a patient with chronic myeloid leucemia). The broken line represents oxygen consumption of lymphocytes (material from a patient with chronic lymphatic leucemia). Oxygen consumed per c.mm. is shown on the ordinate scale.

When cells are collected from anesthetized animals, we have observed that although slight ether anesthesia (30 minutes) does not impair the respiratory process, a longer anesthesia (2 hours)

lowers and even inhibits respiration of leucocytes *in vitro*. Ether does not affect glycolysis when administered for short periods, but when the anesthetic acts for some hours even glycolysis is lowered.

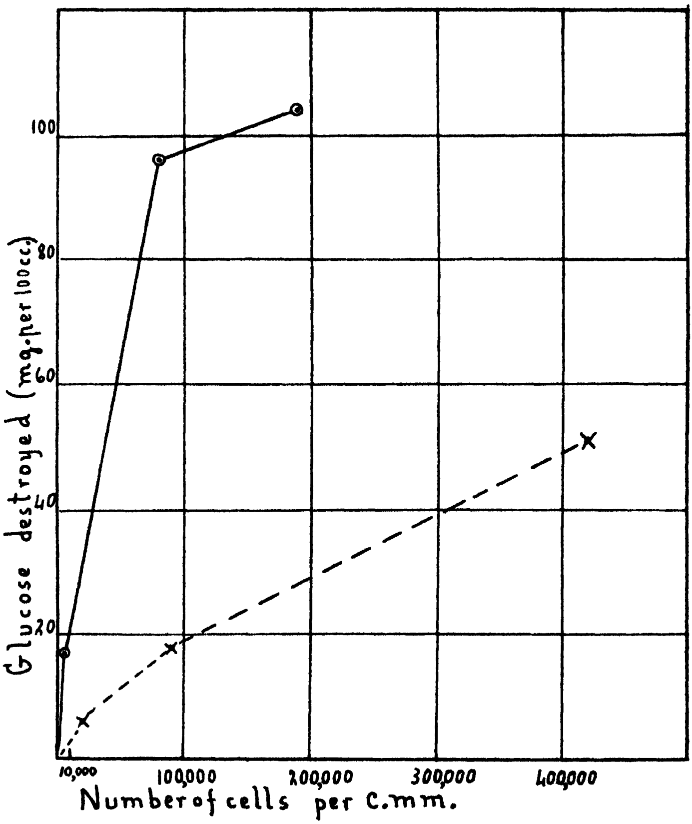

CHART 2. Variation in the glucose consumption of leucocytes at different concentrations. The solid line represents glycolysis of granulocytes; the broken line, glycolysis of lymphocytes. The material was obtained from the same sources as in Chart 1.

Amytal and urethane also inhibit both processes. A detailed report of these effects on blood cell metabolism will be made later.

It was only after much data had been obtained from the study of blood from patients with leucocytosis and leucemia, as well as

from experiments with dog lymphocytes, that a fact of importance emerged whose significance we believe has been neglected in many previous studies; this is, that cell concentration has a very marked influence on metabolic activity. When the oxygen consumption is calculated per million of cells per c.mm. and per unit of time, $K = \frac{\text{mm.}^3\ O_2}{\text{cells (millions)} \times \text{time (hours)}}$, it tends to be in inverse ratio to the cell concentration present. This depressing effect of concentration upon respiratory activity was found to affect the process of glycolysis as well. An example of experiments upon blood cells obtained from a common source, at different serum dilutions, is shown in Charts 1 and 2. It is indicated clearly that the optimum concentration for maximal respiration and maximal glycolysis of both granulocytes and lymphocytes is approximately 10,000 cells per c.mm.

Since normal blood generally contains from 65 to 70 per cent of polynuclear leucocytes and from 25 to 35 per cent of mononuclear cells, it was decided to use only those specimens of blood which contained an excess of one or the other type of cell which it was desired to study Aside from the blood of patients with lymphatic leucemia, lymph from the thoracic duct of dogs furnishes the best material for the study of the metabolism of lymphocytes. This can be obtained by cannulation of the dog's thoracic duct, provided the anesthetic employed for the operation is not harmful to the cells.[2]

Methods.

The methods used were as follows: Blood was collected in purified heparin[3] and centrifuged at once for 2 to 3 minutes. The supernatant fluid was pipetted off, a portion taken for cell counts and smears, and the rest used immediately without being permitted to cool. Oxygen absorption was measured in the Barcroft-Warburg manometers at 37.5° and glycolysis and lactic

[2] That dog lymph contains practically only lymphocytes has been shown by Rous, Davis, and Carlson, and others (Bloom, W., *Arch. exp. Zellforsch.*, 5, 269 (1928)).

[3] This heparin is of a high degree of purity and contains no inorganic salts. It was furnished to us through the kindness of Professor W. H. Howell.

acid formation in flasks kept at the same temperature in a constant shaking device. For anaerobic glycolysis either KCN was added to one flask or purified nitrogen was passed through the flask during the experiment, as indicated in Table I. The sugar was determined by Benedict's modification of the Folin-Wu method and the lactic acid by the Friedemann-Shaffer method,

TABLE II.

Oxygen Consumption of Leucocytes, with Effects of Overcrowding.

Source of material.	*No. of cells per c.mm.	O_2 consumption per hr.	K*	Differential count.
				Polynuclears.
		c.mm.		per cent
1. Chronic myelogenous leucemia.....	48,000	46	0.96	90
2. " " "	72,000	60	0.84	92
3. " " "	162,000	127	0.78	90
4. " " "	123,000	100.6	0.82	90
5. " " "	448,000	268	0.60	91
6. Empyema.......................	30,000	39	1.30	95
7. Pneumonia.......................	30,000	41.4	1.38	84
8. Empyema.......................	28,400	43.0	1.52	86
9. Hodgkin's disease (?)..............	75,000	55	0.73	93
				Lymphocytes.
1. Chronic lymphatic leucemia........	150,000	60.0	0.40	95
2. " " "	52,800	56.2	1.06	90
3. " " "	320,000	54.0	0.017	97
1. Dog lymph lymphocytes...........	10,000	25	2.5	98
2. " " "	8,000	23	2.87	92
3. " " "	6,000	12.5	2.08	93

$$^{*}K = \frac{\text{c.mm. } O_2 \text{ consumed}}{\text{cell concentration (millions)} \times \text{time (hours)}}.$$

before and after incubation. This seemed a more direct, accurate, and specific technique than that used in earlier studies (Fleischmann and Kubowitz (8), Fujita (9)), with the Warburg manometers, and we believe more accurately determines the lactic acid production as a result of glycolysis. From comparative studies with whole blood (erythrocytes do not respire appreciably) we were able to convince ourselves that these manipulative pro-

cedures, including this brief period of centrifuging, do not affect the oxidative metabolism of leucocytes.

In human blood, the oxygen consumption of polynuclear and mononuclear leucocytes seems to be roughly of the same magnitude, with the single difference that polynuclear leucocytes resist overcrowding better than do mononuclear leucocytes (Table II and Chart 1). Doubtless the greater resistance of the glycolytic function to overcrowding is of value in the metabolism of pus cells, for in pus pockets the oxygen supply must be deficient and the cells must depend almost entirely on anaerobic processes of

TABLE III

Aerobic Glycolysis Calculated per Hour and per Million Cells, Showing Effects of Overcrowding upon Sugar Utilization

Experiment No.	Source of material	No. of cells per c mm	Glucose destroyed per million cells per hr
			mg
1	Chronic myelogenous leucemia	48,000	6.25×10^{-3}
2	" " "	72,000	3.45×10^{-3}
3	" " "	162,000	2.95×10^{-3}
4	" " "	123,000	4.76×10^{-3}
5	" " "	448,000	1.32×10^{-3}
6	Empyema	30,000	7.68×10^{-3}
7	"	28,400	1.05×10^{-2}
8	Hodgkins disease (?)	75,000	8.24×10^{-3}
9	Chronic lymphatic leucemia	150,000	5.84×10^{-4}
10	" " "	25,000	2.0×10^{-4}
11	" " "	320,000	2.19×10^{-4}

metabolism for their source of energy. Dog lymphocytes showed a decidedly higher oxygen consumption than lymphocytes obtained from cases of leucemia, but the concentrations used were optimal. The figures in Table II are taken only from those experiments where very slight ether anesthesia was employed.

In their behavior towards glycolysis there is an undoubted difference between polynuclear leucocytes and mononuclear leucocytes. Polynuclear leucocytes possess a higher aerobic and anaerobic glycolysis than mononuclear cells. This is clearly shown in Table III, where the aerobic glycolysis of each variety of cell had been calculated per million cells and per hour of incuba-

tion. In this table it is seen that polynuclear leucocytes appear to have a glycolytic power at least 5 times as great as that of mononuclear cells if we compare experiments in which the concentration is approximately the same (for example, Experiments 3 and 9, and 7 and 10). We have found that polynuclear leucocytes have a higher anaerobic metabolism than was observed by Fujita. It is possible that the explanation of this discrepancy lies in the fact that Fujita was working with a mixture of both polynuclear leucocytes and mononuclear leucocytes. Since lymphocytes have the higher aerobic metabolism, a mean value would thus be obtained under such conditions. Most of our cell preparations have been studied for motility with the dark-field technique and it seems clear that oxygen consumption is greatest in actively motile cells. On the other hand glycolysis and lactic acid formation, and the power of the cell to reduce methylene blue are unrelated to the motility of the cell, for these properties remained unaltered in mature granulocytes in which motility had entirely ceased.

The fundamental researches of Pasteur on "la vie sans oxygen" established the fact that fermentative and oxidative processes are not independent, but closely related. If a cell which ferments sugar in anaerobiosis is put in oxygen, the respiration thus established has a tendency to lower or replace completely the fermentation. Warburg (10) has recently applied Pasteur's fundamental ideas to his classification of the metabolic activity of cells. He properly names as the "Pasteur reaction" this relationship between respiration and fermentation. Taking the maximum value obtained when the cells are put in a condition of optimum oxidative activity and comparing them with those obtained under anaerobic conditions, he calculates the "excess of fermentation" by the following equation.

$$U \text{ (excess of fermentation)} = Q_{\mathrm{M}}^{\mathrm{N_2}} - (2\, Q_{\mathrm{O_2}})^*$$

* $Q_{\mathrm{M}}^{\mathrm{N_2}}$ is the lactic acid produced in anaerobic conditions; $Q_{\mathrm{O_2}}$ is the oxygen consumption.

Since U is the excess of fermentation which remains when the Pasteur reaction reaches its maximum effect, it will be nil when the anaerobic fermentation is twice the respiration, and it will be

TABLE IV.

Calculation of Value for Pasteur Reaction (Warburg) for Granulocytes and Lymphocytes.

Diagnosis.	Time of incubation.	Glucose, mg. per cc. serum.			O_2 consumption, c.mm. calculated for time of incubation.*	Pasteur reaction.	Percentage of cells.
		Before incubation.	After incubation.	Glucose consumption.			
	hrs.						
Chronic myeloid leucemia	3	1.10	0.25	0.85	138	+0.48	90 granulocytes (30% mature polynuclears).
" " "	2½	2.42	1.62	0.80	150	+0.40	92 " (50% " ").
" " "	2	2.45	0.90	1.55	254	+0.87	90 " (40% " ").
" " "	2¼	1.59	0.19	1.40	228	+0.79	90 " (20% " ").
Empyema	2	1.26	0.74	0.52	78	+0.31	95 " (all " cells).
Polycythemia vera	2	2.28	1.10	1.18	140	+0.805	88 " (86% " ").
Chronic myeloid leucemia	1½	1.84	0.25	1.59	402	+0.513	91 " (all " ").
Pneumonia	2	1.04	0.25	0.79	82.8	+0.566	84 " (" " ").
Empyema	1½	0.91	0.37	0.54	65.0	+0.366	86 " (" " ").
Hodgkin's disease	1¼	0.97	0.17	0.80	111.0	+0.50	93 " (90% " ").
Chronic lymphatic leucemia	3	0.83	0.58	0.25	180.0	−0.235	95 lymphocytes.
" " "	2	1.05	0.78	0.27	112.4	−0.03	90 "
" " "	1½	0.75	0.42	0.33	150.0	−0.07	97 "

* 1 c.mm. O_2 = 1.34×10^{-3} mg. of glucose.

negative or positive when twice the respiration is greater or smaller than the anaerobic fermentation. Normal organs, the retina excepted, give negative values under such conditions, while tissues from malignant tumors give positive values for *U*. According to Fleischmann and Kubowitz, blood cells give positive *U* values, thus being similar to the cells of the retina. As seen from Table IV, the present study shows that the values for granulocytic cells are positive and thus indicate that their metabolism resembles that of tumor cells and the cells of the retina. On the other hand, the lymphocytes from cases of chronic lymphatic leucemia all gave negative values, low it is true, but corresponding to the behavior of the cells from normal tissues.

DISCUSSION.

The difference in the anaerobic metabolism (glycolysis) of granulocytic and of lymphocytic leucocytes seems to afford some substantiation to the view of Sabin (11) and her coworkers, based on anatomical grounds that the two types of cells have different origins as well as functions. Glycolytic activity seems to be a more stable type of activity and its greater magnitude in the polynuclear leucocyte must be vital to the activities of this cell when it is placed, as must often be the case, in conditions of very low oxygen tension.

Our results give no support to the suggestion, particularly made by Daland and Isaacs (12), that the respiratory activity of mature polynuclear cells is higher than that of the immature cells. We are unable with our present methods to find any distinct differences in the metabolic activities of immature and mature leucocytes. This fact, in addition to the further observation that the leucocytes from the blood of patients with leucemia do not differ in their metabolism from the corresponding cells of blood from non-leucemic patients, seems to us to indicate that in leucemia we are not dealing with cells with pathologically altered metabolism, such as are found in cancer tissue. Leucemia is not analogous to cancer, at least as regards cell metabolism. It rather seems to be a condition in which large numbers of cells, normal as regards their fermentative and respiratory activities, are thrown out into the circulation as a result of a disturbance in the mechanism which regulates the rate of blood cell formation.

SUMMARY.

1. The basal optimal conditions required for measurement of the metabolism of the white cells of the blood *in vitro* have been defined. These consist primarily in measurement at low concentration, measurement over short intervals of time, use of anticoagulants which do not damage respiration, very short centrifugation, and avoidance of any cooling.

2. No differences have been found in the metabolism of mature and immature granulocytes, or of granulocytes from cases of leucocytosis and of myeloid leucemia.

3. Measured under comparable conditions of concentration, lymphocytes differ in their metabolism from granulocytes in the following particulars: (*a*) The glycolysis of polynuclear leucocytes is about 5 times greater than the glycolysis of lymphocytes. (*b*) There is no marked difference in the oxygen consumption of these two kinds of leucocytes (human) when measured at low concentration, but lymphocytes show a diminution of respiratory power as a consequence of overcrowding more easily than do polynuclear cells. (*c*) The Pasteur reaction in lymphocytes resembles that in normal tissues; the Pasteur reaction in granulocytes resembles that in tumor cells.

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SOME PHYSIOLOGICAL ASPECTS OF COPPER IN THE ORGANISM.

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Copper is so universally distributed throughout the plant and animal kingdoms as to raise the question whether it may not play some important rôle in the metabolic reactions of the organism. Certainly its presence in plants and animals alike cannot be explained simply by its adventitious occurrence in the soil. There is increasing evidence that, up to a certain point, copper may be beneficial to both animals and plants, but that, in excess of this quantity, ill effects sometimes, but not always, ensue. This is particularly suggestive in view of the fact that, with our more accurate methods for the determination of copper, it has been found that the normal quantity of the metal present in plants and vegetables is actually less than that reported in the literature. Investigations have shown that many of the suggested colorimetric methods for the determination of copper will give a high figure if traces of iron are present. Yet it is extremely difficult to eliminate all traces of this metal from biological materials. Our findings have led us to suspect that the amount of copper present bears a definite ratio, not to the quantity of copper present in the soil, but to some compound in the plant. Tschirch (1) has shown that during the artificial greening process a certain amount of copper is first taken up by the chlorophyll, forming a compound called by him phyllocyanate of copper. The excess copper salt then combines with the proteins of the vegetables, as copper always does when it comes in contact with protein. The toxic property of very small quantities of copper to minute organisms was early recognized, especially by von Naegeli (2). This oligodynamic effect is not peculiar to copper, but has been found to occur with other metals, including iron. Naegeli also noted that if materials such

as paper, wool, gum, etc., were added to the solution, this toxic property apparently disappeared. The suggestion has been made that the metal, being present in colloidal form as hydroxide or carbonate, is electropositive and combines with electronegative surfaces of the added material. This, however does not appear to be the whole explanation. We have observed that the amount of protein present affects the toxicity of the copper. This fact was previously observed by Voegtlin (3) who noted that a solution containing a quantity of copper toxic to one tadpole would apparently lose its toxic effect if a larger number of tadpoles were present in the vessel at the same time. It is our belief that in this case the mucin-like substance given off by the tadpoles unites with the copper salt. One of us (Inouye (4)) has shown the affinity of mucin for heavy metals. We have observed that this mucin-like substance given off by Amphibia will remove the copper from a solution, provided the quantity of copper present is not too great.

We found that 0.2 gm. of a mucin-like material excreted by cryptobranchs will remove 10.8 mg. of copper from a solution containing 75 mg. of copper per liter. A close examination shows that if copper or any other heavy metal is present in water containing Amphibia, the mucin-like material given off at the gills of the Amphibia coagulates at the point of excretion. The metal has a greater affinity for newly excreted material than for that which has been floating about in the solution for a time. If sufficient metal is present, the gills become blocked by the coagulated material, in which case the animal dies from suffocation, not from the ingestion *per se* of the copper or other metal. Similarly, we believe that the mucin in man's body is one of his greatest protections against metallic poisoning. In fact, observations indicate that, in the case of Amphibia, the addition of small amounts of metal to the water stimulates the formation of mucin.

Copper has a close affinity for all proteins. In our work we have injected copper salts subcutaneously into animals, and found that these combined with the proteins at the point of injection, and only a very small percentage of the injected copper is recovered in the excreta and body organs. The mass at the point of injection became necrotic and later sloughed off. Only a small amount of copper was absorbed by the blood. Four rabbits received, at 3 day intervals, six subcutaneous injections of 100 mg. of copper

chloride in 10 cc. of physiological saline solution. At the end of 30 days, the animals were killed. The total excreta during this period were collected, and only 9 mg. of copper were recovered. An examination of the organs other than the liver was practically negative. The livers contained on an average 0.6 mg. of copper, a normal amount. A rough analysis of the necrotic area indicated that the copper had been retained at the point of entry. If the copper salt during one of these injections happened to come in contact with an important organ or nerve center, death or paralysis ensued.

Bacteria in water are destroyed if the water flows over copper; but if milk is passed over copper in the same way, the bacterial count is only slightly affected. This protective action of protein is evident in our feeding experiments. A cat which could not take in one dose *per os* 10 mg. of copper chloride without vomiting, was able to ingest 50 mg. in 24 hours without any discomfort, if this was taken with the food. The same observation has been made in respect to other animals. We have also injected 10 mg. of copper chloride, dissolved in 10 cc. of physiological saline solution, into the intestinal tract of a cat. The intestinal tube became contracted, peristaltic action increased, the animal vomited, and an evacuation of the bowels took place. At autopsy the mucosa of the intestinal walls was found to be stained green by the copper salt, this green compound being readily removable. Here again we feel that the mucin-like material acted as a protective agent and prevented corrosion of the walls of the intestines. If food is present in the stomach when copper is fed, the copper tends to unite with it, leaving the mucosa of the gastrointestinal tract unaffected. The copper, combined with the food, is dissolved slowly, at a rate depending on the pH of the stomach or of the intestinal contents. The protective action of food in this connection will be discussed by us in another paper.

We were led to study some of the physiological effects of copper because of Mallory's (5) suggestion that hemochromatosis may result from the continuous ingestion of small amounts of this metal. In a private communication he affirms that in order to produce hemochromatosis, it is necessary to feed the animal more copper than it can assimilate and that the excess copper causes hemolysis, with a subsequent deposition of the blood pigment in

the liver. He believes that anything causing hemolysis will produce such lesions. If Mallory is correct in his belief that copper is a hemolytic agent if taken *per os* and therefore the causative agent of such lesions, it is difficult to explain why hemochromatosis is not found among lead workers, where an anemia occurs which is due to the fragility of the red cells resulting from exposure to lead. Recent work by Flinn and von Glahn (6) indicates that the pigmentation found in the liver of rabbits was not due to copper but was of an exogenous origin.

Elimination and Distribution.—It was necessary in our work to determine the excretion rate and the distribution of the metal in the body of animals subjected to known amounts of copper. For this purpose we made use of the albino rat, kept in a specially constructed metabolism cage so arranged as to permit a separation of the urine and feces and their immediate removal. The copper was given in the drinking water, in the form of copper chloride. The water was placed in a graduated tube, closed at the top, and with a constricted opening at the bottom so arranged as to reduce spillage and evaporation to a minimum.

Copper Assay.—The material was carefully ashed at low temperature in an electric oven, completely dissolved in concentrated nitric acid, and then evaporated to white fumes after the addition of a few cc. of concentrated sulfuric acid. The residue was dissolved in boiling water and the copper plated out on a platinum cone for about 24 hours by means of an electric current. When copper ceased to be deposited, the cone was removed and the copper dissolved from it in a small amount of concentrated nitric acid. The cone was then returned to the original solution and the electroplating continued for another 24 hours. The solution containing the copper dissolved from the platinum cone was evaporated down to about 0.2 cc. and made alkaline with ammonia. From the depth of the blue color, the amount of copper was estimated. The result was checked by the thiosulfate method, with 0.005 N solution of thiosulfate, according to the standard procedure for the determination of the amount of copper by this method.

Twenty-four rats were kept under observation for 82 days, at the end of which time they were removed from the copper exposure; 4 days later they were killed and the intestines removed.

The results are given in the accompanying tabulation. Early in

	mg.
Total copper placed in drinking water in 82 days	1203.5
" " recovered from feces	1020.78
" " " " urine	12.36
" " " " bodies	11.34
" " deposited in drinking tubes	144.00
Total recovered	1188.48

our work we discovered that some of the copper which we thought was ingested was being deposited in the water tubes near their outlet. Probably this was partly precipitated by the mucin of the mouth or by small quantities of food remaining there.

That part of the copper recovered from the feces had been absorbed into the body and then excreted is indicated by the following experiments. A cat was anesthetized and an incision made through the rectus muscle and peritoneum into the abdominal cavity. The gallbladder was brought through the wound and the serosa of the bladder stitched to the peritoneum of the wound. The tip of the gallbladder was then brought to the surface, incised, a drain inserted, and the muscosa of the bladder stitched to the skin with the drain *in situ*. Absorbent cotton was placed over the wound in such a way as to absorb the excreted bile. The dressing was removed each day and analyzed for copper. The cat was given daily doses of 20 mg. of copper chloride in gelatin capsules. An assay of the dressing for each of the first 2 days showed an excretion in the bile of 0.24 mg. of copper. This rate of excretion gradually rose, until at the end of 4 days it reached a level of 1 mg., where it remained for 5 days, when the experiment was terminated by the death of the cat.

Distribution.—In order to study the distribution of copper in the body, we exposed ten rats for 12 months to an average dose of 2 mg. of the metal per day, this being placed in the animals' drinking water. At the end of this period the rats were killed and the intestinal tract of each removed. The findings are given in Table I.

We have repeated this work, not only with rats, but with guinea pigs, and the results confirm the general findings reported in Table I. It can be seen from this table that the liver is the chief depository for the metal.

We have analyzed the livers of various animals that have had no known copper exposure except that contained in their normal food. Table II presents some of our results.

An examination of thirty-three normal human livers, obtained from routine necropsies, showed a copper content of from 2.4 to 15 mg. per 100 gm. of tissue. The livers of 1 day old animals often contain as much copper as those of their parents. The average

TABLE I.

Copper Recovered from Bodies of Rats after 12 Months Exposure to the Metal.

	Cu per gm dry tissue.		Cu per gm. dry tissue
	mg.		*mg.*
Brain	0.21	Kidney	0.14
Bones	0.0046	Liver	0.25
Hair	0.024	Lungs	0.18
Heart	0.05	Muscles	0.008
		Spleen	0.04

TABLE II.

Copper Recovered from Livers of Animals with No Known Copper Exposure.

	Cu per 100 gm. tissue.		Cu per 100 gm. tissue
	mg.		*mg*
Cat	0.23	Guinea pig	3.1
Cow	2.6	Norwegian rat	3.3
Cryptobranch	1.8	Rabbit	1.12
Dog	0.68	Sheep	0.98
Gila monster	1.74	Turtle	8.26
Goat	15.6		

copper content of the livers of 11 guinea pigs, which had been fed 14 gm. of copper daily for 12 months, amounted to 3.7 mg., while for the fetus our findings were 2.9, and for the 1 day old guinea pigs, 2.84 per 100 gm., the figures for all three classes being strikingly similar.

The presence of copper in the hair is interesting because of its occurrence in the highly colored feathers of certain birds, as, for example, the Donaldson touraco, where the red primary feathers contain a pigment, 7 per cent of which is copper. Copper is not

responsible, however, for the "green hair" of copper workers. If we judge from our experimental work, the combination in which copper is normally held in the hair of organisms exposed to this metal is of such a nature as to prevent this weathering effect. Hair from copper-fed animals did not turn green when it was wet with saline solution and permitted to stand exposed to the air.

The smallness of the amount of copper deposited in the bones is a matter for surprise, because we find it stated in the literature that: "Copper dust, or alloys rich in copper, generally produce in workers green-stained hair, greenish deposits on the teeth, and a green tint to the perspiration, which may persist even after a thorough bath, while the skin may be actually bronzed. Some dentists have reported a peculiar purple color and swelling of the gums with pyorrhœa and more or less stomatitis in copper workers. Copper has been found accumulated in all of the organs of the body, in the various excretions, and in the bones of former copper workers, which have shown a green color when subsequently removed from the graves" (7). The question of the deposit of copper in the bones is of special interest to the industrial physiologist, in view of the work of Aub and his colleagues (8), in which they attempt to explain why lead is deposited chiefly in the bones. Copper forms an insoluble phosphate at the same hydrogen ion concentration as lead. One might be justified from this fact and from Fairhall and Shaw's (9) results in inferring that the phosphate is precipitated at an optimum hydrogen ion concentration, and that the same factors that control the deposition of calcium in the bone control this deposition.

In our work, 900 cc. of a solution containing 1 mg. of copper in the form of chloride per 10 cc., were flowed continuously for 30 minutes over 100 gm. of calcined bone by means of an air lift. Before the air entered the air lift, it was passed through $CaCl_2$, KOH, and H_2SO_4. At all times the bone was covered by 10 inches of the solution. A precipitate began to form almost from the beginning of the experiment, a large portion of it settling around the bone, thus giving the impression that the copper salt was combining with the bone. When the bone was washed, however, this proved not to be the case. At the end of 30 minutes the solution was carefully poured off and filtered. The bone was then washed with a high pressure stream of water, in order to

resolve the adhering precipitate. This precipitate was kept separate from the first precipitate, obtained by filtering the solution. For the sake of comparison, this experiment was repeated with a solution containing lead chloride instead of copper.

In another series of experiments, we used fresh bone from which the fat and the protein had been removed first by ether and then by boiling in salt solution until the solution gave a negative biuret reaction. Our results may be summarized as follows: Our analyses do not show replacement of the calcium in the bone by the metal. The precipitate is formed, in our opinion, because of a

TABLE III.

Action of Bone on Metals in Solution as Chlorides.

Bone	Solution. Mg. of metals.	Precipitate.	Metal in filtrate.	Metal in bone.	Ca in filtrate	pH of original solution	pH of filtrate
		mg.	*mg*	*mg.*	*mg.*		
Calcined.	91.8 Cu.	87.4		0.20	4.48	4.5	7.4
	91.8 "	62.1	27.9	0.10	3.75	4.5	6.9
	92.3 Pb.	90.4	0.99	0.23	4.65	6.2	8 2
	92.3 "	91.00	0.82	0.26	4.45	6.2	10.0
Green.	97.2 Cu.	11.9	85.3	0.15	3.45	4.5	6 1
	97.2 "	14.5	83.7	0.10	4.05	4.5	6.2
	125 Pb.	47	76.63	0.08	10 98	6.2	7.1
	125 "	48	86.4	0.15	12.15	6.2	6.6
Calcined.	Water alone.				1.74		10.0

No PO_4 was found in precipitates.

change in the pH of the solution. An analysis of the copper and lead precipitates and solutions after the experiment did not reveal the presence of any phosphates. As a control, water which did not contain either copper or lead salts was flowed over similar portions of bones. Calcium went into solution whether copper or lead was present or not. Our experiments do not confirm the work of former investigators in this respect, nor do they give any indications as to why lead is deposited in the bones of the living organism, while copper, except in traces, is not. Table III shows our results.

Copper is found in normal blood as well as in the blood of animals which have been exposed to this metal. Krebs (10) has en-

deavored to show some connection between different diseases and the copper content of the blood. To study the distribution of copper in the blood, we took an anesthetized cat and injected into an isolated segment of the intestine 10 cc. of a physiological saline solution containing 100 mg. of copper. After a period of from 45 to 60 minutes, a sample of blood was collected by means of a cannula inserted in the carotid artery. This was centrifuged and the plasma separated from the corpuscles. The corpuscles were then washed five or six times with physiological saline solution, by means of the centrifuge. Both plasma and corpuscles were analyzed for copper. Table IV shows our findings.

In Experiments 1 and 2, the solution remained in the segment 45 minutes before the blood samples were collected. In Experiments 3 and 4 the time allowed was 60 minutes. These findings

TABLE IV.

Distribution of Copper in Blood.

Experiment No.	Cu per 100 gm. plasma.	Cu per 100 gm. corpuscle
	mg.	*mg.*
1	1.61	0.99
2	1.33	0.98
3	2.03	1.08
4	2.18	1.11

were confirmed by an examination of the blood of four dogs which were being fed each day 300 mg. of very fine copper in capsules. The average distribution of the copper was found to be 2.97 mg. per 100 gm. of plasma and 1.27 mg. per 100 gm. of corpuscles.

During our investigations as to the effects of various copper salts on the blood *in vitro*, we were led, because of a suspicion that methemoglobin was being formed, to make a spectrophotometric examination of a blood sample. Because of the suggestive readings which were obtained we began to make a study of the blood changes occurring in the living animal receiving copper, particularly with reference to the possible formation of methemoglobin. We made use of the usual type of short haired mongrel dog, kept on a diet of dog biscuit with a little raw meat once a week. These animals were kept under observation for 2 months before the experiment began, during which time the blood was

examined at regular intervals. At the end of this period, the dogs were given daily 300 mg. each of metallic copper, in gelatin capsules wrapped in a small piece of meat. This they readily took at one dose.

The blood was examined for methemoglobin by the Van Slyke-Neill method. In this, one sample of blood, Sample A, received sodium hydrosulfite to reduce the methemoglobin to hemoglobin.

TABLE V.

Examination of Blood of Animals for Methemoglobin by Van Slyke-Neill Method.

	Sample A.	Sample B.
	Dogs fed 300 mg. Cu daily.	
	vol per cent	*vol per cent*
Normal	26.96	27.16
2 wks.	27.12	31.02
2 mos.	31.00	31.74
3 "	32.09	34.35
	Dogs fed 300 mg. aluminum daily.	
	25.74	25.67
	Goats fed lead.	
	23.88	23.90
	Lobster blood.	
Sample 1	3.05	3.04
" 2.	2.49	2.59

A second sample, Sample B, is run without the addition of hydrosulfite. Both samples are saturated with illuminating gas, and the difference in the results should give the amount of methemoglobin in the original sample. In this case results with Sample A should be higher than with Sample B. Table V shows our findings. For comparison, we have included figures for animals that had been fed aluminum and lead, as well as lobsters, in which the iron of hemoglobin has been replaced by copper.

The figures for the copper-fed dogs offer at first sight the ap-

parent anomaly of a part being greater than the whole, since according to the theory of this method of analysis Sample A should give higher figures than Sample B, if methemoglobin is present. A spectrophotometric examination did not reveal the presence of that compound. The evidence tends to show that the copper in the blood is in some combination which is acted upon by the hydrosulfite, and that this combination reacts differently from the compounds which aluminum and lead form in the blood, or from the hemocyanin of the lobster blood. The animals being dosed with aluminum and lead did not show any increase in the oxygen-carrying capacity of the blood, such as the copper animals evidenced.

It has been shown by the diagram prepared by Hurter (11) that no metal except copper forms two oxides and two chlorides in

TABLE VI.

Sample B, Examined for Methemoglobin by Van Slyke-Neill Method.

The results are expressed in volumes per cent.

Dog 1.	Dog 2.	Dog 3.
34 12	29.95	34.47
35.15	26.41	33.85
35.41	28.04	33.24

which the combination is of so loose a character. It is therefore an ideal catalytic agent for certain industrial processes, except for the fact that it is easily poisoned by SO_3, SO_2, and arsenic acid.

CO has a high affinity for reduced hemoglobin. The combination of CO and CuCl is a loose reversible union. This is also apparent in the analysis of the blood samples. There is no difficulty in getting results which check with Sample A when it is reduced or acted upon by the sodium hydrosulfite. But the results with Sample B are not uniform, as is shown in Table VI. The fact that these figures show variation in the different determinations made on the same sample of blood, points to a loose reversible union similar to that which takes place between CO and CuCl. It is this proportion of the blood copper content that is acted upon by the sodium hydrosulfite. The copper is perhaps present in the blood as a catalytic agent, and may be poisoned or

rendered inactive in the same way that CuCl is nullified in industrial plants by SO_3, SO_2, or arsenic. That the variations in our determinations cannot be due to errors in analytical work, is demonstrated by the concordant results obtained with other samples, as shown in Tables V and VI.

We have found no evidence in our work that copper ingested by the body in the normal way acts as a hemolytic agent. Two groups of ten guinea pigs were kept under observation for several

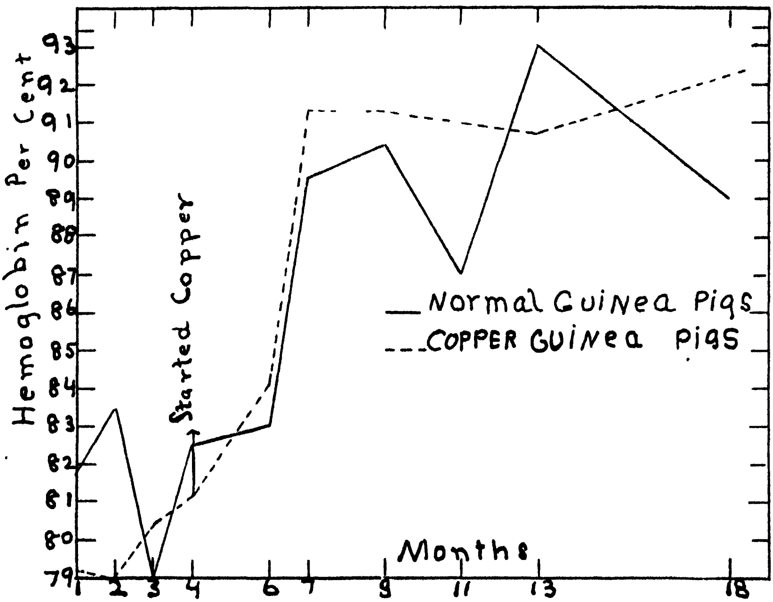

FIG. 1. Effect of copper intake on the hemoglobin of guinea pigs.

months before being given copper. These animals were fed hay and oats *ad libitum*, and in addition on alternate days were given carrots and cabbage. One group was used as a control, while the other group was fed 0.2 mg. of copper in a gelatin capsule, in the form of cupric chloride mixed with lactose. The capsule was placed in the pharynx of the guinea pig and the animal held until the capsule was swallowed. At the end of 5 months, the copper dosage was changed to 1 mg. of the very fine metallic copper, so fine as to pass through silk cloth. Before beginning this work, we

ascertained that the body fluids would dissolve the fine copper. Our investigation revealed that 7.4 mg. of fine copper in contact with 100 cc. of plasma will be dissolved by the end of 24 hours.

During the period that copper was being fed, a blood count and hemoglobin test were made each month. Changes in weight were also carefully noted. The hemoglobin was determined by means of a Dare hemoglobinometer which was standardized from time to time by the Van Slyke method, and the necessary corrections made. Figs. 1 and 2 show the changes that took place in hemoglobin and weight.

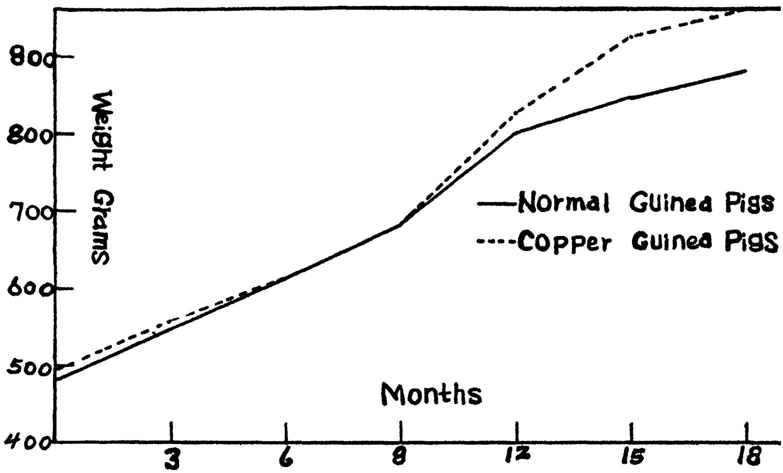

FIG. 2. Effect of copper intake on growth curve of guinea pigs.

The average higher weights of the copper group above that of the control group are not accidental. Our work has consistently confirmed this result. An analysis of the livers of these guinea pigs showed an average content of 0.432 mg. of copper, or 1.66 mg. per 100 gm. of tissue. We have been unable to find, in any of our experimental animals, an increase in the storage of copper in the liver, except in cases where the animals have been fed far larger amounts of copper per kilo of body weight than man would normally be exposed to, even temporarily, in his daily or industrial life.

Accumulation in the Body.—The results of our experiments

indicate that copper may play some important rôle in stimulating blood formation and that its constant presence in the liver, even in the liver of the fetus, may not be due merely to the fact that this organ has among its functions that of being a filter. What the combination is in which it is held, we cannot tell at this stage of our work, but it certainly must exert an important influence on the hematopoietic system and on the metabolism of the body as a whole. An examination of the blood of our guinea pigs and dogs showed that the oxygen-carrying capacity of the blood was increased when copper was being fed to the animals.

We wish to thank Dr. Barbara Stimson and Miss M. C. Hrubetz for making the Van Slyke determinations for us.

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IRON IN NUTRITION.

X. THE SPECIFICITY OF COPPER AS A SUPPLEMENT TO IRON IN THE CURE OF NUTRITIONAL ANEMIA.*

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In articles published from this laboratory (1, 2) it has been shown that the anemia produced in young rats by a diet of whole milk was not cured by the addition of pure iron salts. It has been further shown, however (3), that the addition of small amounts of a copper salt along with the iron brought about a prompt cure of the anemic condition. Since copper is not apparently a constituent of the hemoglobin molecule and since, therefore, it serves to increase the hemoglobin formation in some indirect way, the question arose as to its specificity in this reaction. Immediately following our discovery of this rôle of copper in nutrition, we undertook, therefore, to see if any other element possessed the property of supplementing iron in the cure of the anemia produced by a diet of whole milk.

In Paper IX of this series (4) we have presented data showing that, in a variety of liver preparations, copper was the only element present that possessed the ability to supplement a basal diet of milk and iron. It is well known that the liver is a storehouse for many of the substances taken into the body and that it contains in a greater or less degree all the elements so ingested. The fact, therefore, that the supplementing effect of various liver preparations was in direct proportion to their copper content offered strong presumptive evidence that this element, among

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† E. R. Squibb and Sons Fellow.

those normally present in the animal body, was entirely specific in its antianemic action.

We have, in addition, during the past year studied the effect of adding to the basal diet of whole milk and iron, pure salts of various elements in an attempt to find out if any possessed the ability to bring about increased hemoglobin formation. In all, twelve elements were studied; namely, zinc, chromium, germanium, cobalt, nickel, tin, lead, cadmium, mercury, antimony, arsenic, and manganese. We sought to include in this group all those elements that are known to be present in natural foods, that have been reputed to have a stimulating action on hematopoietic function, or that are chemically similar to copper. Thus zinc and manganese, and in lesser degree, cobalt and nickel, are found widely distributed in various plant and animal tissues and several attempts have been made to elucidate their function in the animal body (5–8). Germanium has been found by some (9, 10) to be a definite stimulant to the production of red blood cells although this has been denied by others (11). Arsenic in small doses has been known to have a stimulating or tonic action on the body in general and has been thought to exert this action on the red marrow of the bones in particular. While continued exposure to lead produces in man the condition of plumbism or chronic lead poisoning, a cardinal symptom of which is a stubborn anemia, it was thought that this element in small doses might show a supplementing effect when added to the basal diet. The other elements included in the group above show some chemical similarity to copper.

EXPERIMENTAL.

Our procedure throughout was to feed standard aqueous solutions (or suspensions) of a pure salt of each element. The choice of the salt was dependent on availability, purity, and solubility. Each element was fed at three different levels, generally, 1.0, 0.1, and 0.01 mg. daily, six times per week. In some cases the dosage was higher, while with definitely toxic elements such as arsenic and mercury, the dosage was one-tenth of the above. In each case the solution of each salt was fed to young anemic rats receiving as a basal diet whole milk and iron.[1]

[1] The basal diet was cow's whole milk fed *ad libitum* plus 0.5 mg. of Fe as ferric chloride. During the period when the animals were being made

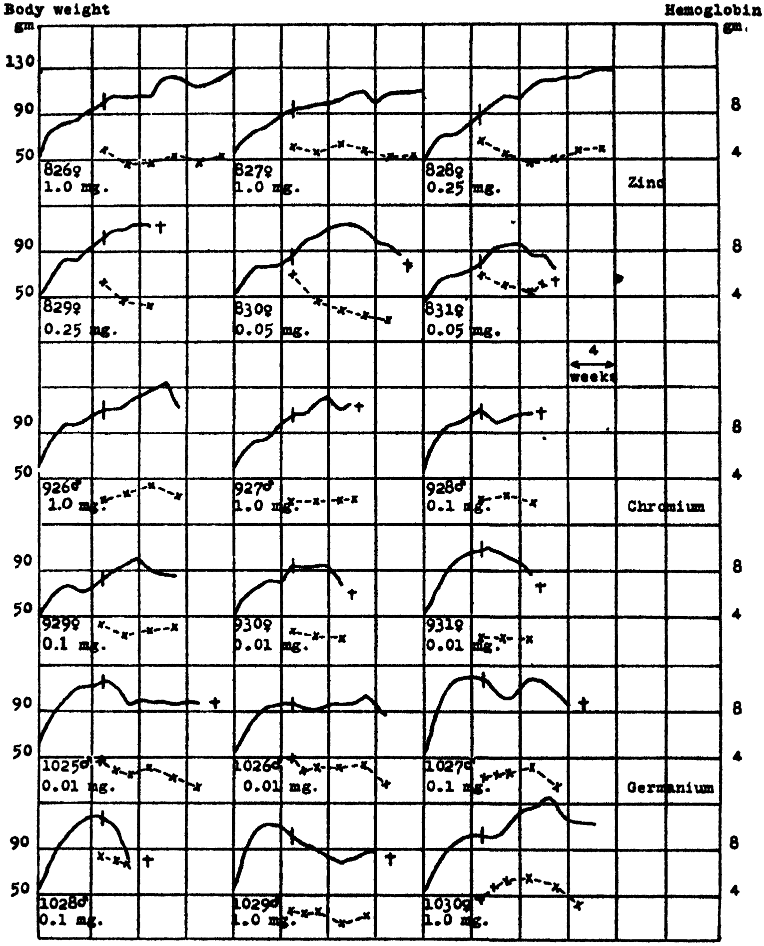

Chart I. Showing the results obtained when either zinc, chromium, or germanium was added to the basal diet of milk and iron. All three elements are quite inert as regards hemoglobin formation on this basal diet.

In this and all the other charts the solid line represents the curve of body weight, while the broken line represents hemoglobin in gm. per 100 cc. of blood. The line across the curve of body weight marks the addition to the basal diet; the dagger indicates death.

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anemic (5 weeks generally) milk alone was fed. The iron and other additions were made to the morning feeding of milk six times per week. More milk was given as this was consumed.

Zinc.

Zinc sulfate (Merck's C.P.) was used for these experiments. Three aqueous solutions were made up such that 1 cc. contained 1.0, 0.25, and 0.05 mg. of Zn respectively. The results secured when these three levels were added to the basal diet are shown graphically in Chart I (Rats 826 to 831). No response in hemoglobin production was secured.

Chromium.

Chromium sulfate (Mallinckrodt's C.P.) was made up in three aqueous solutions such that 1 cc. contained respectively 1.0, 0.1, and 0.01 mg. of Cr. These three concentrations of this salt were fed to anemic animals receiving the basal diet of milk and iron. As may be seen in Chart I (Rats 926 to 931), no improvement in hemoglobin levels was secured. At the end of 5 weeks four of the six animals were dead.

Germanium.

Germanium dioxide (Eimer and Amend) was dissolved in water by the aid of heat and the addition of several drops of dilute NaOH. Three solutions were made up and fed at levels of 1.0, 0.1, and 0.01 mg. of Ge daily six times per week. The results, also shown graphically in Chart I, were entirely negative.

Beard and Myers have recently reported (12) that the addition of germanium and iron to rats made anemic on milk brought about more prompt cures than the addition of iron alone. We cannot confirm these findings and have already suggested (2) that their iron may not have been copper-free. Whipple and Robscheit-Robbins (13) have failed to secure any favorable effect from feeding germanium to dogs made anemic by bleeding.

Nickel.

Nickel sulfate (Merck's Highest Purity) that had been in the laboratory for some years and had lost some water of crystallization was recrystallized from water. After drying the salt between filter paper, three solutions were made up and the three levels of 1.0, 0.1, and 0.01 mg. of Ni were fed to anemic rats. The results, as may be seen in Chart II, were uniformly negative.

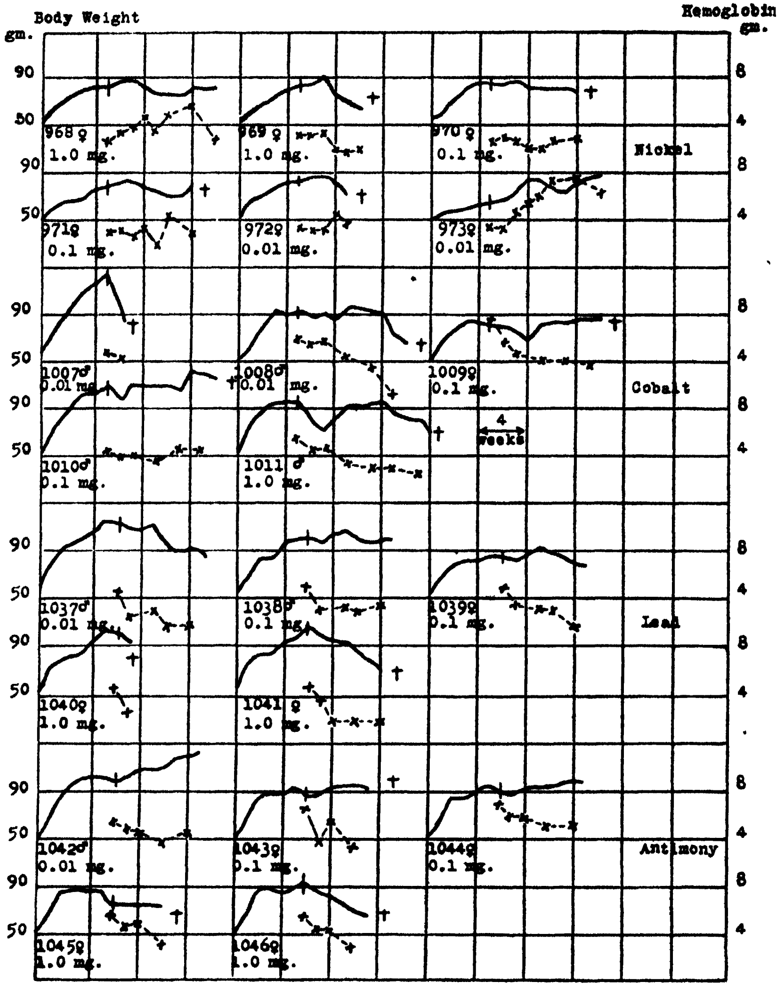

CHART II. Showing the effects of adding to the basal diet of milk and iron either nickel, cobalt, lead, or antimony. None of these additions brings about hemoglobin increases on this diet.

Cobalt.

Cobaltous chloride (Baker and Adamson, Analyzed) was made up in standard aqueous solutions and the three levels of 1.0,

0.1, and 0.01 mg. of Co were fed. As may be noted in Chart II (Rats 1007 to 1011) no improvement in hemoglobin titer was obtained.

With regard to the favorable effect of nickel and cobalt which was reported by Beard and Myers (12) our previous criticism on germanium is pertinent.

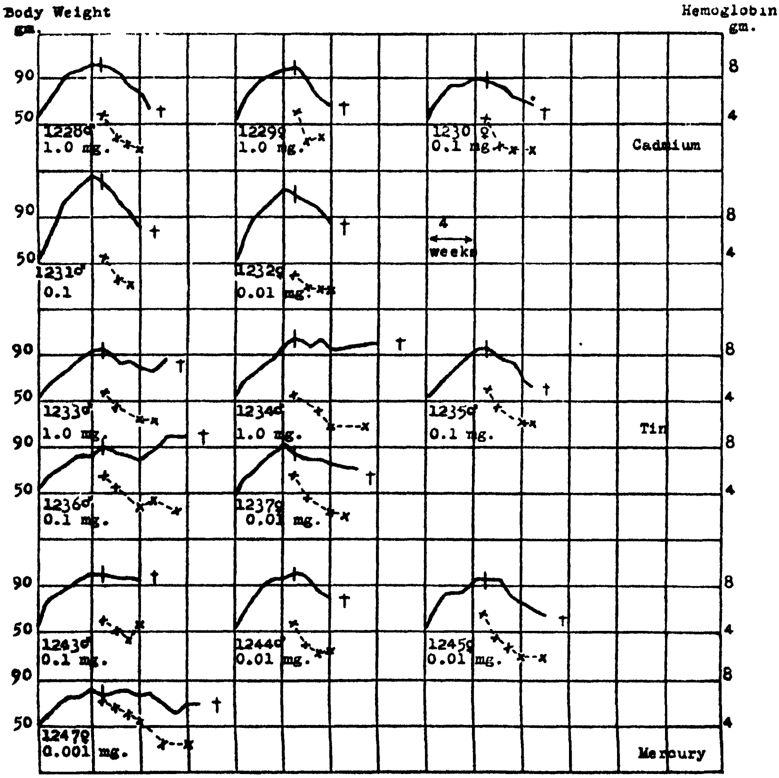

Chart III. Showing the ineffectiveness of either cadmium, tin, or mercury to supplement a basal diet of milk and iron.

Lead.

Lead acetate (Baker Chemical Company, U.S.P.) was used in this experiment. The three levels of 1.0, 0.1, and 0.01 mg. of Pb were fed as aqueous solutions with the entirely negative results shown in Chart II (Rats 1037 to 1041).

Antimony.

Antimony chloride (Mallinckrodt's C.P.) was used here. This salt hydrolyzes immediately in water, and hence, in order to get a clear solution, crystalline tartaric acid (Mallinckrodt's C.P.) was added to the antimony chloride in a small amount of water. Only as much as was needed to effect solution was added. This was then diluted to volume. The three levels of 1.0, 0.1, and 0.01 mg. of Sb were fed and the results are shown in Chart II (Rats 1042 to 1046). Here again the antianemic effect was entirely negative.

Cadmium.

The salt used in this experiment was cadmium chloride (Mallinckrodt's C.P.). Aqueous solutions were added to the basal diet in amounts such that 1.0, 0.1, and 0.01 mg. of Cd were introduced. The results (Chart III, Rats 1228 to 1232) show that cadmium had no supplementary effect as regards hemoglobin formation.

Tin.

For the experiments with tin, stannic chloride (Baker and Adamson's C.P.) was used. This was made up in three aqueous solutions such that 1 cc. represented respectively 1.0, 0.1, and 0.01 mg. of Sn. The two stronger solutions did not remain clear and were consequently fed as suspensions, being vigorously shaken each time. These three levels were fed to two groups of anemic rats. The results obtained with one group are shown in Chart III (Rats 1233 to 1237) and it may be noted that no improvement in hemoglobin production was obtained. The second group showed an equally poor response.

Mercury.

Mercurous sulfate (Mallinckrodt's C.P.) was dissolved in water with the aid of a few drops of pure sulfuric acid and was fed to anemic rats at three levels. Because of the marked toxic properties of mercury the levels used were 0.1, 0.01, and 0.001 mg. of Hg per rat daily (six times per week). These three levels were fed to two groups of animals with entirely negative results. The results with one group of four animals are shown in Chart III. The results with the other animals were exactly similar.

Arsenic.

For our experiments with arsenic we made use of arsenic trioxide, As_2O_3 (Mallinckrodt's U.S.P.). By the addition of a small amount of pure HCl to a weighed amount of the oxide in water, solution was secured. The levels fed were 0.1, 0.01, and 0.001 mg. of As. These amounts were first fed to a litter of six anemic rats. Two of these died within the 1st week after the additions were made to the basal diet and are not included. The results

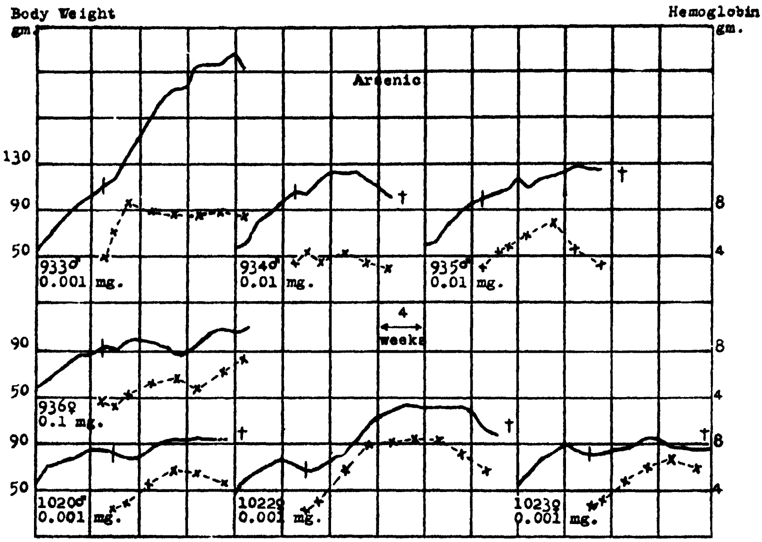

CHART IV. Showing the results secured when arsenic was added to the basal diet. The effect is only temporary and minimal.

secured with the remaining four animals are shown in Chart IV (Rats 933 to 936), where it may be noted that one animal (Rat 933) showed some stimulation to hemoglobin formation. The response of this rat to the arsenic addition was an increase of slightly more than 4 gm. of hemoglobin during the first 2 weeks and the maintenance of a hemoglobin titer of close to 8 gm. per 100 cc. of blood for a period of 10 weeks. The growth increases of this animal were also marked. The other three animals showed insignificant responses, two dying within 8 weeks after the addition was made.

Because of the rather favorable reaction of Rat 933 to the addition of 0.001 mg. of As, this level was again fed to a group of three rats with the results shown in Chart IV (Rats 1020, 1022, and 1023). Here it may be noted that all three showed a slight increase in hemoglobin formation for several weeks, this being most marked in the case of Rat 1022, but that they all eventually suffered a falling off in hemoglobin titer and all died. The response of Rats 1020 and 1023 is not very significant, owing to the absence of growth increases. However, from the record of these two rats, taken in consideration with the records of Rats 933 and 1022, it would appear that the addition of very small amounts of arsenic has a slight stimulating effect on hematopoiesis. However, this effect is not marked and appears to be transient and cannot be compared to the results secured with the addition of copper. It may be noted that the postmortem findings in Rats 1020, 1022, and 1023 showed a gross pathology similar to that found in animals dying from anemia, on the basal diet. The outstanding gross findings are greatly dilated heart, the ventricular musculature being much thinned, edema of the lungs, and effusion of fluid into both pleural and peritoneal cavities. The mucous membranes are pale and the liver is also generally pale, the lobules being visible to the naked eye.

In a further experiment we sought to determine whether arsenic added to the basal diet along with copper would show greater supplementing effects than where copper was added alone. Consequently in a group of four animals, two received as an addition to the basal diet of milk and iron, 0.001 mg. of As and 0.01 mg. of Cu, while the other two received the 0.01 mg. of Cu alone. Over an experimental period of 20 weeks, no differences were noted, the response in body weight and hemoglobin increases being remarkably uniform. For economy of space these records are not included in the charts.

Manganese.

One of the first elements to be studied in this series of experiments was manganese. The salt that we used was manganese chloride (Merck's Highest Purity) and it was fed at levels of 5.0, 0.25, and 0.01 mg. of Mn. These levels were added to the basal diet of a litter of anemic rats with the results shown in Chart V

(Rats 915 to 919). There it may be noted that one of the animals receiving the 5.0 mg. of Mn level died after 3 weeks. The four remaining animals were continued on this diet for a period of 12 weeks, at the end of which time one died. The response in hemoglobin formation was very meager, but the slight upward trend of

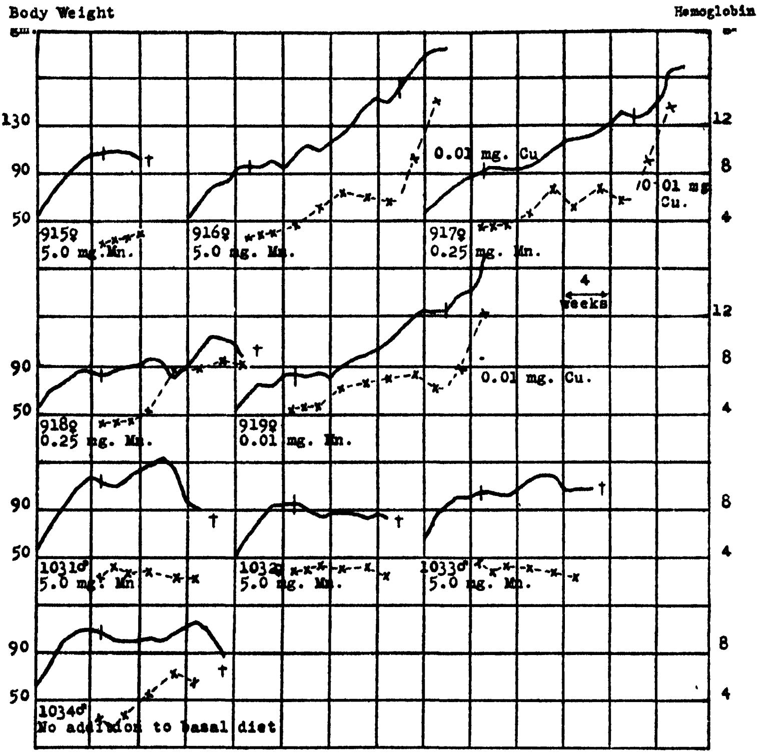

CHART V. Showing the results of adding to the basal diet various levels of manganese. In Rats 916, 917, and 919 the second addition was 0.01 mg. of Cu which replaced the manganese addition.

the hemoglobin curves, together with the slow growth maintained in the three that survived, suggested that either the manganese was bringing about some stimulation or that the animals had good bodily reserves and were responding temporarily to the iron in the diet. We decided therefore to carry out more experiments on this

manganese salt. Before discarding the three animals in this group we wished to see if they would still respond to copper, and, therefore, discontinued the manganese additions and substituted 0.01 mg. of Cu as copper sulfate. The response was immediate and marked, as may be seen in Chart V.

In the second experiment carried out with manganese we made use of the highest level since we wished to secure a maximum effect. Three animals in a group of four received the manganese addition while the fourth received only the basal diet of milk and iron. The results secured are shown in Chart V (Rats 1031 to 1034). The manganese additions were entirely impotent. It is of interest to note that the animal on the basal diet showed a slight response in hemoglobin before succumbing. This observation again emphasizes the fact that some individuals possess sufficient reserves to respond slightly and temporarily to the iron of the basal diet.

About the time that the above experiments on manganese were concluded Dr. Titus of Kansas Agricultural College in a personal communication informed one of us of the results of his experiments. The conclusions of Titus and his associates, whose paper appeared later (14), were that manganese was able to bring about increases in hemoglobin quite comparable to those secured when copper was added to a diet of milk and iron, and that the addition of both manganese and copper was more effective than either alone. While our own experiments had indicated that manganese alone was quite inert, we thought that the combination of manganese and copper might possibly be more effective than copper alone. We therefore fed to three of a litter of five anemic rats 0.01 mg. of Cu and 0.25 mg. of Mn, while the other two received only the copper. Over an experimental period of 20 weeks no differences could be noted in either hemoglobin response, body weight increases, or in general physical well being between the animals receiving both manganese and copper and those receiving copper alone.

In addition to the above experiments we carried on others, using the manganous carbonate which had proved effective in the experiments of Titus, Cave, and Hughes. We secured this salt through the courtesy of the above authors, who were kind enough to send us a generous sample of it, and prepared a solution of

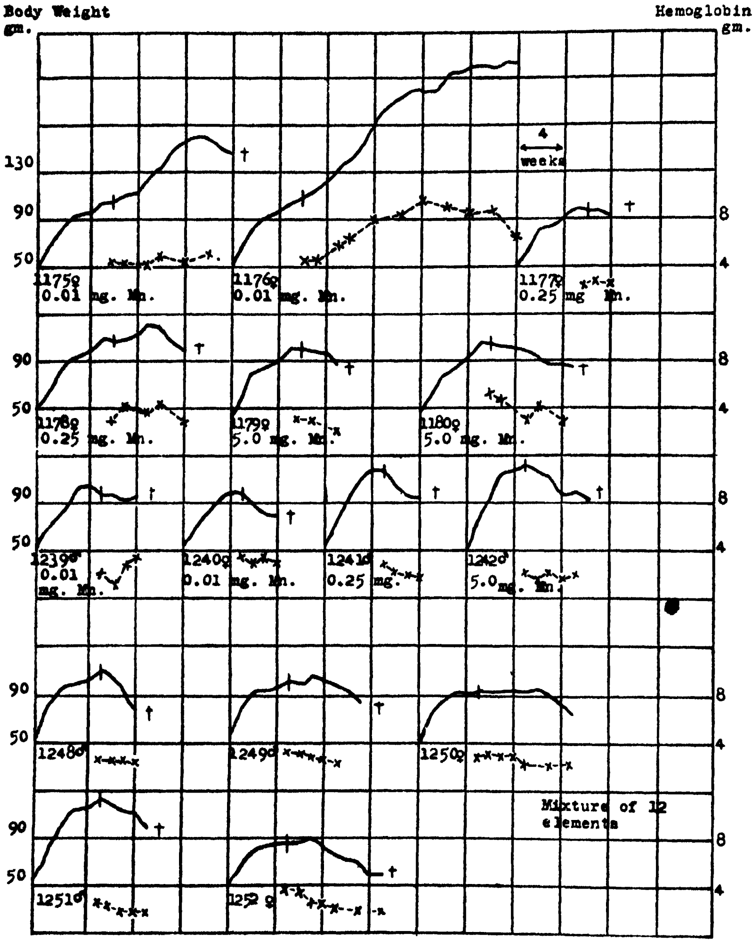

Chart VI. In this chart are shown the results secured when there were added to the basal diet the various levels of manganese, the salt secured from Titus and associates being used (Rats 1175 to 1180, Rats 1239 to 1242). In the lower portion of the chart are shown the results of adding to the basal diet a mixture containing all twelve elements discussed in this paper.

manganese chloride by treating the carbonate in the manner described by them. We added this preparation to the basal diet at the same levels we had previously used, namely, 5.0, 0.25,

and 0.01 mg. of Mn, using in all thirteen anemic rats. The results obtained were in all cases but one entirely negative. In Chart VI we present the records of ten of these animals. The only individual which showed any response was Rat 1176. This animal responded slowly over a relatively long time, the hemoglobin rising to between 9 and 10 gm. at the highest, and being maintained above 8 gm. for a period of approximately 12 weeks. The growth was also good. We cannot explain the performance of this animal unless by assuming that it possessed sufficient reserves to tide it over the experimental period. In all other cases, as we have stated, the results were entirely negative, being typified by the records of the other animals in Chart VI, which received manganese.

Mixture of Twelve Elements.

Having concluded all of the experiments which we have described above, in which each of the twelve different elements had been tested as to its ability to supplement milk and iron in the cure of nutritional anemia, as a final experiment we prepared a mixture of all twelve elements and added this to the basal diet. This mixture was made by taking a portion of a solution of each salt, such that 1 cc. of the final mixture contained, of each element, the lowest level at which it had been fed previously. To the basal diet of a litter of five anemic rats 1 cc. of the mixture was added per rat daily, 6 times per week. The results, as may be seen in Chart VI, were entirely negative.

DISCUSSION.

It is clear from the experiments that we have described that none of the twelve elements studied is able to replace copper in the cure of the anemia produced by the basal diet. All of them, with the possible exception of arsenic, are distinctly negative in their effect and arsenic brings about only a temporary and minimum response in hemoglobin production. Copper is, therefore, quite unique in its action and must be considered a necessary element in animal nutrition.

The work of Beard and Myers (12) and Titus, Cave, and Hughes (14) has tended to indicate that copper was not the only element that possessed the ability to bring about increased hemoglobin

formation in animals fed whole milk and iron. We have already offered a possible explanation of the results of the first-named authors. We cannot explain the findings of Titus, Cave, and Hughes, since they apparently guarded against copper contamination in the various additions to the milk diet. This is a matter of the utmost importance, since, as we have demonstrated in previous publications, exceedingly small amounts of this element bring about marked responses. It may well be that manganese (or any of the other elements discussed in this paper), is necessary for normal nutrition of the animal body and for the production of hemoglobin in particular, but we have not been able to demonstrate it unequivocally in animals confined to our basal diet, even when using the same manganese salt used by Titus and his associates.

Whipple, Robscheit-Robbins, and their associates (15) have emphasized the fact that the anemia which they study is different from ours. We too wish particularly to emphasize this point. We make use of a diet which is deficient, a deficiency which we have been able to show is relatively simple. They, by excessive hemorrhage, bring about a deficiency in their experimental animals, which is undoubtedly multiple in nature. The use of a diet which is admittedly complete for normal life processes, together with the loss of many substances to the body by hemorrhage, seems too complicated for the elucidation of the rôle of any one substance in hematopoiesis. This would seem to be particularly true if the substance in question were necessary only in very small amounts. It is to be expected, therefore, that their evidence would point to a "group of substances" (organic and inorganic) rather than to one substance as being responsible for increased hemoglobin formation. They have reported that while iron and copper had a stimulating action, iron alone was more effective than copper alone. The need for iron is undoubtedly greater than the need for copper, since it is a constituent of the hemoglobin molecule and since by bleeding it is lost to the animal body. In view of the fact that their basal diet undoubtedly contains some copper it is not surprising that iron would have a pronounced effect. We would, in addition, suggest that the "salt effect" or "catalytic action" of *high iron dosage* observed by them may have been due to small amounts of copper contained in their

iron. We have already shown (2) that in our studies with rats high iron intake does not increase hemoglobin production above that obtained with smaller doses when the iron is carefully freed from its copper contaminant.

Although it would seem that cow's whole milk plus an addition of iron and copper is a complete diet for the rat as far as hemoglobin production is concerned, it remains to be seen whether this diet can successfully support all the other functions of the body. Krauss (16) has reported that he was unable to secure reproduction on such a diet. We have been working on this problem for some time and shall report on it in a future publication.

SUMMARY.

1. Twelve elements, in addition to copper, have been studied as to their ability to supplement a diet of whole milk and iron in the production of hemoglobin in the rat. These elements included zinc, chromium, germanium, nickel, cobalt, lead, antimony, tin, cadmium, mercury, arsenic, and manganese.

2. None of these twelve elements has been found to approach copper in its ability to bring about increased hemoglobin formation. All, with the possible exception of arsenic, are quite inert. The effect of arsenic is minimal and temporary.

3. It appears, therefore, that copper is unique in this connection and must be considered a necessary element in the nutrition of the animal body.

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THE RELATION OF IRON AND COPPER TO HEMOGLOBIN SYNTHESIS IN THE CHICK.*

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Shortly after we had demonstrated in this laboratory (1) that nutritional anemia could be induced in rabbits by a diet of cow's whole milk, and that this anemia could not be corrected by inorganic iron unless it was supplemented with a natural food material, or a preparation from this material, we attempted to use chicks as experimental animals for anemia studies. A survey of the literature at that time indicated that chickens had been used rarely for studies of this nature. The only work found was that of Coppela, as he is quoted by Stockman (2), who was able to reduce the hemoglobin in cocks to 33 per cent by feeding a diet containing no iron and to increase it, in 5 days, to 65 per cent by the addition of ferric lactate.

We were interested in using chicks because they are readily available laboratory animals and because we wished to obtain results with animals other than rabbits. Since that time rats have also been used in this laboratory (3) and it was the work with this animal which demonstrated the importance of copper as a supplement to iron for hemoglobin building (4).

In this paper we wish to present some of the fundamental facts observed during the work with the chicks, and to give the results which demonstrated that copper has the same favorable effect upon the hemoglobin synthesis in the chick as it has in the rat.

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EXPERIMENTAL.

Day old chicks were brought into the laboratory and placed in pens that were properly warmed and fitted with wire screen bottoms to prevent consumption of any refuse. White Leghorns were used almost entirely in all the work. The first group was placed on a diet of cow's whole milk, but, although there was a noticeable decrease in the hemoglobin content of the blood, the chicks were unable to consume enough of the liquid milk to obtain sufficient nourishment. An attempt was made to eliminate this difficulty by supplementing the liquid milk with whole milk powder. This also was found to be unsuccessful because normal growth was not maintained. Therefore, it was decided that some food material which possessed considerable bulk must be added to the milk diet. Polished rice was chosen as the supplement because the iron content of rice is lower than that of any of the other cereals. The addition of cracked rice to the ration stimulated growth, but the anemia developed rather slowly. Finally it was found that if the rice was previously extracted with hot alcohol, this difficulty was eliminated. The rice was extracted in large percolators with alcohol at 37° for a period of 7 days. The alcohol was changed daily. The rice was then dried and 97 parts of the rice mixed with 2 parts of $CaCO_3$ and 1 part of NaCl. This mixture, together with cow's whole milk fed *ad libitum*, constituted the basal ration. The chicks were also irradiated for a period of 10 minutes every other day to insure the prevention of rickets.

When day old chicks were kept on this ration, they invariably developed anemia in 12 to 15 days. The amount of hemoglobin decreased from the normal of about 8 gm. per 100 cc. of blood to about 4 gm. per 100 cc. Samples of blood for hemoglobin determinations were obtained by puncturing one of the veins on the under-side of the wing. The hemoglobin was determined by the Dare hemoglobinometer in the earlier work and later the Newcomer method was used. When the Dare instrument was used, the figure for the gm. per 100 cc. of blood was calculated by multiplying the reading in percentage by the standard of 13.77 gm. given for this instrument. When the Newcomer method was used, the figure for the gm. per 100 cc. was obtained directly from the colorimeter reading.

It is not surprising to find this rapid drop in the hemoglobin content of the blood of chicks placed on a ration low in iron, because the reserve supply of iron in a chicken when hatched is very low. The iron content of an egg of average size is about 0.8 to 1.0 mg. of Fe. The amount present in the entire body of a chick at birth

TABLE I.

Hemoglobin Content of Chick Blood as Modified by Ferric Oxide and Lettuce Ash.

Chick No.	Diet.	Days on diet. 0	5	10	15
		Hb per 100 cc. blood.			
		gm.	*gm.*	*gm.*	*gm.*
202	Basal.	5.2	4.8	4.1	3.2
203		4.4	3.2	2.7	1.6
204		3.4	2.5	2.5	1.8
205		3.8	3.2	2.7	3.4
206		5.4	4.0	3.2	2.9
Average.		4.4	3.5	3.0	2.6
208	Basal + Fe_2O_3 equal to 2 mg. Fe per chick per day.	2.7	3.2	3.3	4.5
209		4.8	4.5	4.5	4.8
210		1.4	2.3	2.1	2.3
211		4.5	3.4	3.7	3.8
212		3.7	4.1	3.8	3.2
Average.		3.4	3.5	3.5	3.7
219	Basal + Fe_2O_3 equal to 2 mg. Fe per chick + lettuce ash equal to 1 gm. per chick per day.	3.8	8.0	8.0	9.6
220		4.1	8.0	7.7	9.6
221		2.7	5.5	6.6	8.0
222		4.3	7.3	8.9	8.3
223		5.1	6.2	6.5	10.3
Average.		4.0	7.0	7.5	9.2

is between 0.6 and 0.7 mg. of Fe, most of which can be accounted for in the hemoglobin of the blood. This shows that the chick has practically no iron store from which additional hemoglobin can be built.

The chicks were allowed to become anemic by feeding them as a group in a large pen on the basal ration for a period of 12 to 15

days. The hemoglobin content of the blood of the individual chicks was then determined, and the chicks placed in small cages, five chicks in a group. Various additions were then made to each group of chicks.

Table I gives typical records of a group of five chicks continued on the basal ration 18 days after the preliminary feeding, a group

TABLE II.

Hemoglobin Content of Chick Blood as Modified by Soluble Iron Salts.

Chick No.	Diet.	Days on diet. 0	6	12
		Hb per 100 cc. blood.		
		gm.	*gm.*	*gm.*
246	Basal.	3.9	3.0	2.8
247		4.9	3.2	3.2
248		3.6	2.8	1.8
249		4.7	3.4	3.4
250		4.9	4.4	3.6
Average...		4.4	3.4	3.0
287	Basal + $FeSO_4 \cdot 7H_2O$, Sample II, equal to 2 mg. Fe per chick per day.	3.6	7.9	8.3
288		4.8	7.9	8.4
289		3.8	6.6	8.5
290		3.8	6.2	7.5
291		4.8	6.6	7.9
Average...		4.2	7.0	8.1
424	Basal + $FeSO_4 \cdot 7H_2O$, Sample IV, equal to 2 mg. Fe per chick per day.	4.9	6.9	6.3
425		4.8	4.8	7.2
426		4.8	6.9	9.2
427		3.7	7.6	8.3
428		3.7	7.4	8.8
Average...		4.4	6.7	8.0

fed Fe_2O_3 with the basal ration, and a group fed lettuce ash in addition to the iron supplement. There is a continual deterioration of the blood stream in the chicks continued on the basal ration. When the chicks are fed ferric oxide alone, there is no increase in the hemoglobin content of the blood. As soon as lettuce ash is added, there is the same characteristic improvement as was noted previously in the work with rabbits (5).

Several groups of chicks were also given iron additions in the form of ferrous sulfate. Whenever this salt was fed, rapid and complete recoveries were made. The records of two groups fed ferrous sulfate are given in Table II. Ferrous sulfate Sample II was made from iron wire and ordinary sulfuric acid. Sample IV was made from iron wire and purified sulfuric acid. These records are typical of all the chicks that received a soluble iron salt. We attempted to prepare the salts in the purest possible form but the source of the iron in each case was iron wire, which might contain small amounts of impurity. Since these salts were all potent, we felt that the rapid response was probably due to the presence of some other element found in the iron salt as a contaminant.

Because ferric oxide did not bring about an improvement, we thought that this salt was free from the contaminating element and could therefore be used together with the basal ration in the determination of the element active in hemoglobin synthesis. A large number of salts of different elements were fed to the basal Fe_2O_3 ration. It is interesting to state that $CuCl_2$ was fed with Fe_2O_3 to chicks as early as April, 1926. No improvement in the blood stream was noted when any of these salts was fed together with Fe_2O_3.

The hemoglobin of all the chicks remained so uniformly low that the probability that this continued anemic condition was due to the lack of an available iron supply presented itself. Perhaps the iron in the ferric oxide was not being utilized by the growing chick. This assumption appeared quite possible since the feeding of a soluble iron salt resulted in an increase in the hemoglobin of the blood.

This fact suggested an experiment which would demonstrate definitely whether the iron in ferric oxide can be utilized by chicks. Three groups of fifteen chickens each were used for this work. One group was allowed to remain on the basal diet after preliminary feeding, one group was given Fe_2O_3 equivalent to 2 mg. of Fe per chick per day, and one group was fed $FeSO_4 \cdot 7H_2O$, Sample XI, at the same level of iron intake. This ferrous sulfate was prepared from electrolytic iron and sulfuric acid. The hemoglobin was followed for 10 days after which time the chicks were killed, the livers removed, dried, and analyzed for iron. The results are given in Table III.

The figures show conclusively that the iron content of the livers from the chicks receiving ferric oxide was no higher than the iron in livers of the chicks on the basal ration. The iron in the livers of the chicks fed the ferrous sulfate was over 6 times as high. The ferric oxide is therefore not assimilated by chicks. This fact explains the impotence of ferric oxide when used as a source of iron. This finding is also of practical importance since it shows that if iron is added to the ration of chicks it should not be in the form of ferric oxide.

The only alternative, therefore, was to use a soluble iron salt in further anemia studies with chicks. Since all the soluble iron salts used up to this time had stimulated hemoglobin regeneration, we turned our efforts to the purification of various iron salts to such an extent that they would not stimulate hemoglobin formation when fed alone. The preparation and purification of iron salts for chicks continued until it was demonstrated by the use of rats that pure iron salts were ineffective in hemoglobin synthesis unless the iron was accompanied by minute quantities of copper.

TABLE III.

Effect of Fe_2O_3 and $FeSO_4$ on Hemoglobin Content of Blood and Iron Content of Liver of Chicks.

Group No.	Diet.	Days on diet. 0	5	10	Iron content of dry liver.
		Hb per 100 cc. blood.*			
		gm	*gm.*	*gm.*	*per cent*
1	Basal.	4.1	3.8	3.7	0.0119
2	" + Fe_2O_3.†	4.0	4.1	4.2	0.0103
3	" + $FeSO_4 \cdot 7H_2O$†, Sample XI.	3.4	6.2	6.6	0.0672

* Figures for hemoglobin and iron content of liver are the averages of the fifteen chicks in each group.

† Iron salts fed at levels equivalent to 2 mg. of Fe per chick per day.

When the pure iron salts which had been found ineffective with rats and which had been found copper-free by actual test, were fed to chicks, a response similar to that noted with all soluble salts was obtained. The iron salt used in most of the work was $FeCl_3$, which was prepared in exactly the same manner as described in a previous publication (6).

In Table IV we present in detail the results obtained by the addition of pure $FeCl_3$ to the basal diet and the addition of pure $FeCl_3$ supplemented with copper. It is readily seen that the pure $FeCl_3$ fed alone stimulated hemoglobin formation as well as the ferric chloride supplemented with copper. The stimulation in

TABLE IV.

Effect of a Pure Iron Salt and This Iron Salt Plus Copper on Hemoglobin Regeneration When Fed with Basal Ration.

Chick No.	Diet.	Days on diet. 0	6	12	18	24
		Hb per 100 cc. blood.				
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
1250	Basal.	3.5	3.1	2.5	2.6	2.4
1251		3.8	3.1	2.6	2.1	Dead.
1252		3.8	3.3	3.2	3.8	"
1253		4.2	3.3	3.3	4.3	3.3
1255		4.0	3.2	2.9	4.2	3.1
Average.		3.9	3.2	2.9	3.4	2.9
1256	Basal + 0.1 mg. Fe as $FeCl_3$ (purified) per chick per day.	2.6	6.0	8.3	7.9	7.6
1257		4.0	6.2	8.3	6.7	Dead.
1258		4.0	6.4	8.1	Dead.	
1259		3.9	4.4	6.4	6.7	6.7
1260		3.8	7.2	8.4	8.3	7.6
Average.		3.6	6.0	7.9	7.4	7.3
1261	Basal + 0.1 mg. Fe as $FeCl_3$ (purified) per chick per day, + 0.01 mg. Cu as $CuSO_4$ per chick per day.	3.8	6.6	6.9	7.9	7.2
1262		3.3	7.0	8.7	8.9	7.8
1263		4.0	7.3	8.6	8.5	8.6
1264		3.8	7.6	7.7	9.1	8.1
1265		2.7	6.5	7.6	7.4	7.3
Average.		3.5	7.0	7.9	8.3	7.8

this case could not be due to any impurity in the iron salt, and we therefore turned our attention to the basal ration. It was thought improbable that the milk could furnish enough copper to effect hemoglobin synthesis because the work with rats had shown that when pure $FeCl_3$ was added to the milk ration of the rat, no regeneration took place. The only other source of copper supply

was from the rice preparation. Since the active element was known to be copper, we analyzed the rice preparation for this element and found it to contain 2.5 mg. of Cu per kilo. If a chick consumed 5 gm. of the rice per day, it would obtain 0.013 mg. of copper, which is a sufficient amount to stimulate hemoglobin

TABLE V.

Effect of a Pure Iron Salt and This Iron Salt Plus Copper on Hemoglobin Regeneration When Fed with a Modified Basal Ration.

Chick No.	Diet.	Days on diet. 0	6	12	18	24
		Hb per 100 cc blood.				
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
1362	Modified basal + 0.2 mg. Fe as $FeCl_3$ (purified) per chick per day.	4.2	3.6	3.6	3.6	2.6
1363		4.3	3.7	2.8	3.3	Dead.
1364		4.2	3.9	4.1	4.4	3.5
1365		4.0	3.6	4.0	4.7	4.9
1366		4.0	4.7	3.7	3.4	Dead.
Average.		4.1	3.9	3.6	3.9	3.6
1368	Modified basal + 0.2 mg. Fe as $FeCl_3$ (purified) + 0.01 mg. Cu as $CuSO_4$ per chick per day.	4.0	4.0	4.7	4.8	4.6
1369		4.2	5.3	6.3	7.4	7.4
1370		4.0	6.6	6.2	6.6	7.2
1371		3.9	5.8	6.4	7.7	7.0
1373		3.8	5.7	7.2	7.2	7.4
Average.		4.0	5.5	6.2	6.7	6.7
1376	Modified basal + 0.2 mg. Fe as $FeCl_3$ (purified) + 0.02 mg. as $CuSO_4$ per chick per day.	4.0	5.6	6.2	6.5	Dead.
1377		3.3	4.2	6.0	7.9	7.0
1378		4.4	5.4	7.2	7.7	7.0
1379		4.8	6.5	6.1	6.4	5.3
1380		4.1	5.3	4.1	4.5	5.2
Average.		4.1	5.4	5.9	6.6	6.1

synthesis. This fact explains immediately the results obtained with all the soluble iron salts. The basal ration was low enough in iron to produce anemia providing no soluble source of iron was supplied, but it was not low enough in copper to prevent hemoglobin building when the iron was supplied in available form.

In order to demonstrate conclusively that copper is the neces-

sary supplement to iron in the case of chicks as well as in rats, it was necessary to construct a basal ration which was practically free from copper. The difficulty of finding any food material other than milk which is exceedingly low in copper is readily seen by a study of the table giving the copper content of food materials which was published recently from this laboratory (7). The only material which looked at all promising was corn-starch. The starch was granulated by making a thick paste and drying at 37°. When this granulated starch replaced the rice in the basal ration, the chicks did not grow. The most decided deficiency of this ration seemed to be a lack of vitamin B because as soon as yeast was added good growth was obtained. Of course, yeast could not be used to supplement the ration since it contains considerable copper. A hot 90 per cent alcoholic extract of yeast also contained some copper. However, if this extract was evaporated to a thick paste, taken up in water, and extracted with ether, the largest portion of the copper was removed with the fat. In this way a fairly concentrated preparation of vitamin B, free from copper, was obtained. When this material, equivalent to 20 gm. of yeast, was added to 100 gm. of granulated starch, the ration so modified gave fair growth.

In Table V are given the results obtained when iron alone and iron and copper additions are made to this modified basal ration. When purified $FeCl_3$ is added to this ration, no improvement in the hemoglobin content of the blood takes place. As soon as 0.01 mg. or 0.02 mg. of copper as copper sulfate is fed each chick daily, a decided increase in the hemoglobin content is noted.

DISCUSSION.

The results presented in this paper verify again the great anemia-producing power, *i.e.*, the low iron and copper content, of whole milk. There seems to be no difference in the animal used; as soon as it is restricted to a milk diet directly after weaning, anemia develops rapidly. However, in the case of an animal like the chick, which will not grow normally on a milk diet alone, the introduction of additional food, unless highly purified, has a favorable effect upon hemoglobin synthesis. These results, as well as actual analyses of these foods for iron and copper, demonstrate the universal distribution of these hemoglobin-building elements in

most natural foods. This fact is of great importance whether one is interested in producing anemia in experimental animals on any diet other than milk, or whether one is interested in the use of natural foods as a supplement to milk for the prevention of anemia in young growing animals.

One must be exceedingly careful in the preparation of food materials other than milk for the production of anemia in experimental animals. Several natural foods may be purified to such an extent that they are low enough in iron to produce anemia, but it is very difficult to reduce the copper content enough to maintain the anemia when sufficient iron is added to the diet. The modified basal ration used in the chick work presented was low enough in copper to maintain anemia when it was supplemented with pure ferric chloride, but the growth of the chick was not entirely normal.

Recently Drabkin and Waggoner (8) reported that the severe anemia developed in rats on a milk diet could be cured by placing these rats on a copper-free synthetic ration. These workers were so kind as to furnish us a sample of their copper-free synthetic ration for analysis. Our analyses show that this ration contained 0.044 mg. of copper and 0.532 mg. of iron per 10 gm. of the ration. Assuming the rats consumed 10 gm. each of the ration per day, they would be ingesting the optimum amounts of iron and copper for hemoglobin regeneration. It certainly would be surprising if a ration compounded of materials such as egg albumin, commercial casein, and dry brewers' yeast did not contain some copper. The importance of careful estimations of copper in anemia-producing diets cannot be overemphasized.

CONCLUSIONS.

Day old chicks placed on a diet of cow's milk together with polished rice, calcium carbonate, and sodium chloride, invariably become anemic. The hemoglobin falls from 8 gm. per 100 cc. of blood to 4 gm. per 100 cc. in 12 to 15 days.

Additions of ferric oxide to this ration will not prevent the anemia because the iron in ferric oxide is not assimilated by chicks.

The addition of ferrous sulfate or purified (copper-free) ferric

chloride to this ration immediately stimulates hemoglobin synthesis because this basal ration contains small amounts of copper.

When purified ferric chloride is added to a modified basal ration very low in copper, no stimulation is noted until minute amounts of copper are added.

Copper acts as a supplement to iron in hemoglobin synthesis in chicks as well as in rats.

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A METHOD TO DETERMINE THE CARBON DIOXIDE CONTENT OF MUSCLE.

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In principle and general construction the apparatus used is precisely similar to the manometric blood gas apparatus described by Van Slyke and Neill (1924), with a few modifications as shown in Figs. 1 and 2. To allow the insertion of the muscle, the burette is made in two separable parts. The upper part, or neck, fits into the lower part, or body. Air-tight union is effected by a ground joint. While the pressure within the burette is subatmospheric the ground surfaces of the neck and body are held tightly together, but when the burette is first being filled, the buoyant force of the mercury tends to lift up the neck. This tendency is opposed by a detachable spring catch, *C*, (Fig. 1) which holds the neck securely in the socket. Two of these burettes are attached to one manometer and one mechanical shaker, allowing two determinations to be made almost simultaneously, on the one apparatus.

Outline of Manipulation.

When readings or adjustments are being made on one burette, the other is cut off from the system by closing its lower stop-cock (Stop-cock 2 or 3 as the case may be). If we are using Burette 1 Stop-cock 2 is open while Stop-cock 3 is closed. By lowering the cistern, *N*, the mercury level is depressed to mark *A* and fixed there by closing Stop-cock 4. The frozen muscle sample and a drop of caprylic alcohol are placed in the body of the burette. The neck is fitted into position and fastened down. By raising cistern *N*, with Stop-cock 4 open, mercury is allowed to fill the burette and overflow into cup *B*, and Stop-cock 1 is closed. 6 cc. of acid placed in the cup above the mercury are run back into the

burette through Stop-cock 1. Stop-cock 1 is again closed and this time sealed with a drop of mercury. By lowering the cistern the mercury meniscus in the burette is brought to *A* again and fixed

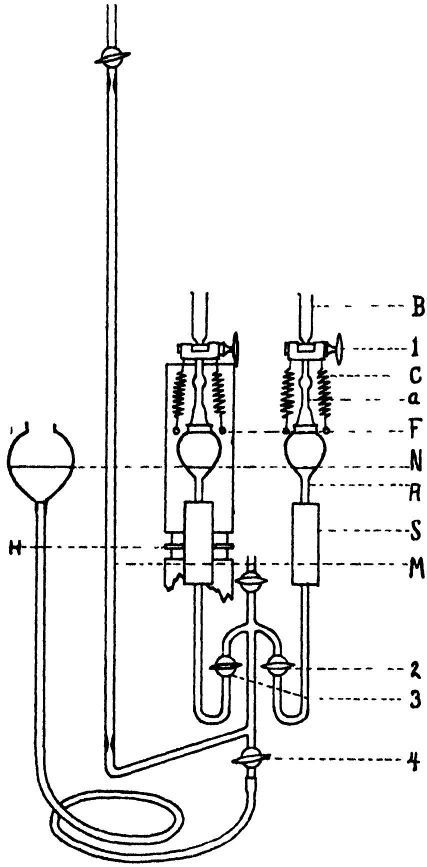

Fig. 1. Two muscle burettes are shown connected with one mercury reservoir and manometer. The spring catch, *C*, is fastened to a wooden frame which supports each burette, but which is represented only behind Burette 2. Above, the springs are attached to two flat hooks which slip over the shoulders of the upper stop-cock.

1, 2, 3, and 4 are stop-cocks; *B*, a graduated cup; *C*, a spring catch; *N*, a mercury cistern; *M*, a manometer tube; *S*, a mercury-sealed flexible joint; *a*, the mark on the burette at 2 cc. from Stop-cock 1; *A*, the mark on the burette at 50 cc. from Stop-cock 1; *F*, a wooden frame; *H*, a hinge.

by closing Stop-cocks 2 and 4. The other burette may now be similarly filled. Both burettes are vigorously agitated by the mechanical shaker for 45 minutes.

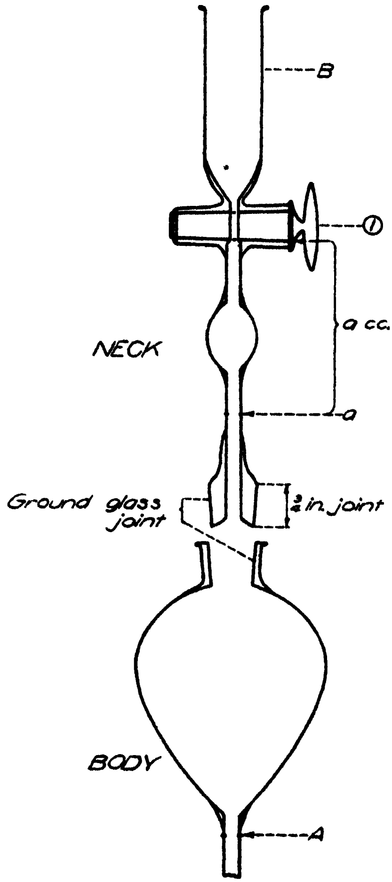

FIG. 2. A diagram of the muscle burette showing the two parts, neck and body, separated. 1 is a stop-cock; *B*, a graduated cup; *a*, the mark at 2 cc. from Stop-cock 1; *A*, the mark at 50 cc. from Stop-cock 1. The measurements of the apparatus are: total length, 30 cm.; bore of tubing, 5 mm.; length of ground joint, 17 mm.; width of ground joint, 20 mm. at center; bore of Stop-cock 1, 2 mm.; length of ground surface of Stop-cock 1, 35 mm.; width of barrel of Stop-cock 1, 12 mm. at center; and capacity of *B* is about 10 cc.

With the cistern in the position shown in Fig. 1, Stop-cock 2 is fully opened, then Stop-cock 4 is cautiously opened, allowing the mercury slowly to fill the burette till the volume of the gases in the burette is reduced to a. Then Stop-cock 4 is again closed. The reading on the manometer scale gives P_1. Now the burette is again evacuated and the CO_2 is absorbed by running 1 cc. of 1.5 N NaOH through Stop-cock 1. When the gaseous volume has again been reduced to a the manometer reading gives P_2. From the difference between P_1 and P_2, we calculate the CO_2 content of the sample.

Sampling.

The muscles are dissected out as expeditiously as possible and immediately dropped into liquid air. While frozen, the muscles are weighed to the nearest mg. Each sample should weigh 1.5 gm. ±0.2 gm. During the weighing it is necessary to reimmerse the muscle in liquid air, and to wipe the condensed moisture from the watch-glass at 2 minute intervals.

Solutions.

We are using 6 cc. of 0.1 N hydrochloric acid to acidify the muscle. Furusawa and Kerridge (1927) have plotted titration curves for skeletal muscle. These show that 1.5 gm. of muscle require 0.14 mM acid to lower the pH from 7.0 to 5.0; *i.e.*, 1.4 cc. of 0.1 N HCl is the minimum of acid required. In using 6 cc. of 0.1 N HCl a safe excess is assured. It is necessary to use at least 6 cc. of solution in order that the muscle may float freely above the mercury. For if the muscle deforms the mercury meniscus an error is introduced into the readings of P_1 or P_2.

1 cc. of 1.5 N NaOH is used to absorb the CO_2. The alkali is rendered air-free in an evacuating bulb. In itself the addition of 1 cc. of alkali to the solution above the mercury causes the mercury to be lower during the reading of P_2 than during the reading of P_1 (Van Slyke and Neill, 1924), therefore $P_1 - P_2$ is slightly greater than the pressure exerted by the CO_2 absorbed. Thus if P represents the pressure exerted by the CO_2, P equals $p_1 - p_2 - c$, where c is a correction determined in blank experiments in which 1.3 cc. of CO_2-free distilled water are substituted for the muscle. In this case the difference between P'_1 and P'_2 can only be due to

the added alkali. Hence c equals $P'_1 - P'_2$. When 6 cc. of acid are used c has a value of 1 mm. in our burettes. The volume of muscle used is not exactly the same each time but these slight variations in the volume (of muscle and acid) produce no appreciable error in c.

40 minutes have been found to be the maximum time required for the extraction of all the CO_2 from the muscle. In practice,

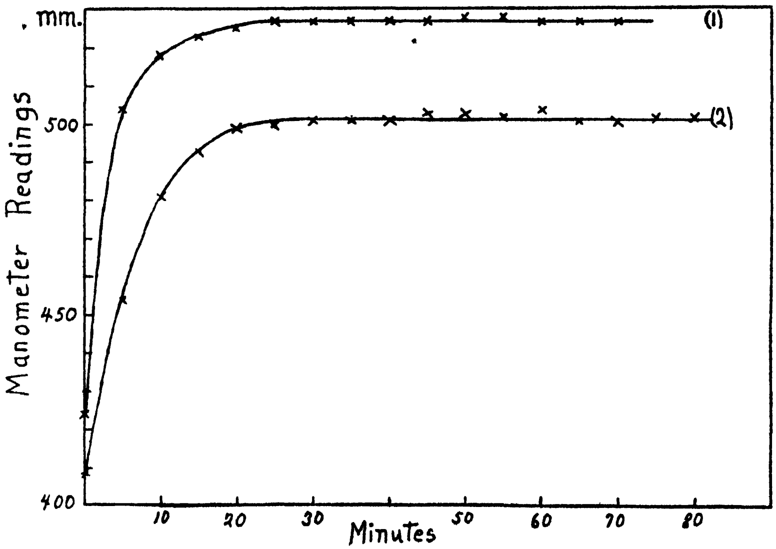

FIG. 3. Every 5 minutes after the commencement of shaking, the gaseous volume was reduced to a, and p_1 read. The values of p_1 are plotted against time, showing the rate of liberation of CO_2 from muscle.

we allow 45 minutes before reading P_1, then shake for 5 minutes more and check the reading of P_1. Fig. 3 represents readings of P_1, plotted against the time of shaking. They not only show the time required for the extraction of CO_2, but being actual readings they show to what extent the apparatus may be regarded as completely leak-proof.

Reabsorption Coefficient.

After shaking, while the gas volume is being reduced to a, a certain proportion of the CO_2 is redissolved in the acid (Van Slyke

and Stadie, 1921). The extent of this reabsorption is determined empirically by the analyses of standard carbonate solutions (Van Slyke and Sendroy, 1927). The results obtained on the muscle burette are multiplied by an empirical factor, i, (the reabsorption coefficient) to make them agree with results obtained by gravimetric and titration analyses of the same carbonate solution.

Calculations.

Van Slyke and Neill (1924) derive the following equation.

$$VCO_2 = \frac{P \times i \times a}{760\ (1 + 0.00384\ t)} \left(1 + \frac{S}{A - S}\,\alpha'\right) \qquad (1)$$

in which

VCO_2 = CO_2 content of sample expressed in cc. at 0° and 760 mm. mercury.
P = $P_1 - P_2 - c$ (c, P_1, and P_2 are defined above).
a = volume between mark a on burette and Stop-cock 1.
t = Centigrade temperature.
A = volume between mark A on burette and Stop-cock 1.
i = reabsorption coefficient (defined above).
α' = $\alpha \times \frac{T}{273}$, where α is the Bunsen solubility coefficient for CO_2 in water at $T°$; and T is the absolute temperature. (α' = Ostwald solubility coefficient of CO_2'.)
S = volume of (blood and acid).

We have had to introduce one more symbol (S') to make this equation applicable to determinations on muscle.
The modified equation reads:

$$VCO_2 = \frac{P \times a \times i}{760\ (1 + 0.00384\ t)} \left(1 + \frac{S'}{A - S}\,\alpha'\right) \qquad (2)$$

The symbols all have the same meanings as before, with these two exceptions: S = the volume of acid and muscle. Thus if we use 1.5 gm. of muscle and 6 cc. of acid, $S = 6 + \frac{1.5}{1.07} = 7.4$ cc. 1.07 is the figure we have found for the specific gravity of cat muscle. S' = the volume of water which, at any given pressure, will dissolve as much CO_2 as 6 cc. of 0.1 N HCl + 1.5 gm. of muscle.

From the work of Van Slyke, Sendroy, Hastings, and Neill (1928)

it would appear that a good figure for α_{CO_2} in whole blood would be 0.483 at 38°.

Now whole blood contains about 79 per cent water by weight while muscle contains about 75 per cent water.

A good assumption for the value of α_{CO_2} in muscle would appear to be $\frac{75}{79} \times 0.483 = 0.46$ or roughly 85 per cent of α_{CO_2} in water at 38°.

We will accordingly assume that α_{CO_2} in muscle is $0.85 \times$ α_{CO_2} in water at the same temperature.

Thus at any pressure of CO_2 (6 cc. of 0.1 N HCl + 1.5 gm. of muscle) will dissolve as much CO_2 as:

$$\left(6.0 + \frac{1.50}{1.07} \times 0.85\right) = 7.19 \text{ cc. water at the same temperature.}$$

$$i.e.\ S' = 7.19 \text{ cc.}$$

According to Van Slyke and Stadie (1921), VCO_2 is the sum of two quantities: (1) V, which is the CO_2 extracted from the sample by shaking; (2) X, the CO_2 still remaining in the solution (and muscle in our case) even after shaking.

From the gas laws

$$V = \frac{P \times a \times 273}{760 \times T} \tag{3}$$

If p^1 is the partial pressure of CO_2 in the extraction chamber immediately after shaking

$$X = \frac{p^1 \times S' \times \alpha}{760} \tag{4}$$

But

$$p^1 = P \times \frac{a}{A - S} \tag{5}$$

Substituting for p^1 in equation 4 we get

$$X = \frac{P \times a \times S'}{760\ (A - S)}\alpha \tag{6}$$

But

$$VCO_2 = V + X = \frac{P \times a \times 273}{760 \times T} + \frac{P \times a \times S' \times \alpha}{760 \times (A - S)}$$

or

$$VCO_2 = \frac{P \times a}{760} \left(\frac{273}{T} + \frac{S' \times \alpha}{A - S} \right)$$

By taking $\frac{273}{T}$ out of the parentheses we get

$$VCO_2 = \frac{P \times a \times 273}{760 \times T} \left(1 + \frac{S'}{A - S} \alpha' \right) \tag{7}$$

because

$$\alpha \times \frac{T}{273} = \alpha'$$

The reabsorption coefficient, i, must now be included, also a correction for the variation—in density with temperature—of the mercury column in the manometer tube (Van Slyke and Stadie, 1921). These are combined with Equation 7 to give the final form

$$VCO_2 = \frac{P \times a \times i}{760\ (1 + 0.00384\ t)} \left(1 + \frac{S'}{A - S} \alpha' \right)$$

Let us see what error is introduced into the factor $\left(1 + \frac{S'}{A - S} \alpha' \right)$ by the assumption that S' and S are constant at values of 7.19 and 7.4 respectively, when really they vary with w, the weight of the muscle, which may weigh 1.5 gm. ± 0.2 gm.

If w is 1.50 gm., the real $S' = 6 + \frac{1.50 \times 0.85}{1.07} = 7.19$; $S = 6 + \frac{1.50}{1.07} = 7.4$.

At 18° α' is about 1.0 while $A = 50$ cc. for our burettes;

$$\therefore 1 + \frac{S'}{A - S} \alpha' = 1 + \frac{7.19}{42.6} = 1.0169$$

If w is 1.3 gm. the real $S' = 6 + \frac{1.30 \times 0.85}{1.07} = 7.03$ and $S = 6 + \frac{1.30}{1.07} = 7.21$.

$$\therefore 1 + \frac{S'}{A - S}\alpha = 1 + \frac{7.03}{42.8} = 1.0164$$

Thus if we assume a constant mean value for S' and S the maximum error introduced is 1 in 2000. If we limit the weight of the muscle samples to 1.5 gm. ± 0.2 gm., we may, like Van Slyke, use a table of factors with temperature as the only variable, to calculate the CO_2 content.

TABLE I.

Analyses of Na_2CO_3 Solutions (Volume Per Cent CO_2).

Date	Gravimetric.	Titration.	Blood burette (Van Slyke).	(i = 1.03) Muscle burette. 1.	(i = 1.03) Muscle burette. 2.
1929					
Jan. 11		88.0	87.6	87.2	
			87.2	87.7	
Mar 8		33.1	33.3	33.4	33.5
		33.4	33 2	33.2	33.1
" 16	103.0	103.1		102.0	102.5
	102 0	102.9		101.5	101.3
" 30	57.3			57.2	57.7

Table I shows that for analyses of carbonate solutions the muscle burette is accurate to within 1 per cent. From Table II it appears that determinations on different cat muscles at the same time, and on different parts of the same muscle, may differ by as much as 5 per cent. We have not come to a definite conclusion as to whether the limiting factor in the accuracy of the method as a whole lies in a real variability of CO_2 content of muscle, or in crude methods of sampling. The average of the percentage deviations from the mean of each pair of determinations is 1.9 per cent in the case of the determinations on two parts of the same muscle, and 1.4 per cent in the determinations on right and left muscles.

Table III has been included to show the CO_2 contents of the

TABLE II.

CO_2 Contents of Muscles.

Measurements are in cc. of CO_2 per 100 gm. of muscle.

Determinations on two parts of same muscle.			Simultaneous determinations on right and left muscles.		
Muscle.	Part 1.	Part 2.	Muscle.	Right.	Left.
Extensor digitorum longus.	34.2	35.0	Gastrocnemius (frog).	25.0	24.3
" " "	18.3	17.0	" "	23.6	24.0
" " "	31.3	32.9	Brachialis anterior.	25.6	25.9
" " "	17.6	18.2	Extensor digitorum longus.	24.3	23.0
Obliquus externus abdominis.	14.5	13.7	" " "	22.8	22.8
Brachialis anterior.	24.1	24.2	" " "	31.3	32.9
" "	21.5	20.5	" " "	18.2	17.6
Tibialis anterior.	20.0	19.5	Tibialis anterior.	19.5	20.0
Average of percentage deviations from mean of each pair of determinations.	1.96 per cent.			1.38 per cent.	

TABLE III.

CO_2 Contents of Muscles of Normal Anesthetized Cats.

Measurements are in cc. of CO_2 per 100 gm. of muscle.

Muscle.	CO_2 content.
Tibialis anterior..	20.0
" "	19.5
" "	23.9
Extensor digitorum longus..........................	26.2
" " "	25.5
" " "	20.1
" " "	19.5
Gastrocnemius..	20.8
Peroneus longus..	19.8
" "	21.6
" "	15.8 Low.
Extensor digitorum longus..........................	16.4
" " "	26.3 High.
" " "	26.1
" " "	17.6
Tibialis anterior..	26.2
Average..	21.6·

resting muscles in eight anesthetized cats. The series is too short for the application of statistical analysis, but it gives a rough idea of the average value and variability of the CO_2 content of normal cat muscles.

We have pleasure in acknowledging the efficiency of the technical work of Mr. F. L. Robinson in connection with this research.

SUMMARY.

A method is described for the determination of CO_2 in muscle samples of 1.5 gm. The muscle is fixed by liquid air and the CO_2 extracted and measured by a method similar to that used by Van Slyke and Neill for blood CO_2.

The probable error of this method is less than 2 per cent.

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THE INFLUENCE OF PROTEIN AND INORGANIC PHOSPHORUS ON SERUM CALCIUM.

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In 1917 Binger (1) demonstrated that elevation of the concentration of inorganic phosphate in the blood by injections of sodium phosphate was attended by reduction of the serum calcium, an observation that was confirmed by Tisdall (2). Subsequent clinical (3, 4) and experimental (5, 6) studies have left no doubt that, other things being equal, the concentration of calcium in the serum and body fluids is dependent upon the level of inorganic phosphate in these media.

Salvesen and Linder (3), in 1923, from a study of the relation of calcium to protein in sera and transudates from patients with nephritis and heart disease concluded that the amount of protein in body media also had an important influence upon the concentration of calcium in these media. Since then Marrack and Thacker (7), Loeb (8–11), and Hastings, Murray, and Sendroy (6) have shown that proteins increase the solubility of calcium in true and artificial sera, probably by the formation of unionized or only slightly ionized Ca-protein complexes.

It follows from these studies that abnormalities of the calcium concentration of the serum can be interpreted with accuracy only if phosphate and protein have been determined simultaneously. Low serum calcium can be considered as evidence of an essential defect of calcium metabolism only if phosphate and protein are normal. If the level of phosphorus or protein is disturbed it is, at present, impossible to estimate the effect of such disturbance

* Part of this work was done by Dr. Leo Eiserson in fulfilment of the thesis requirement for the Degree of Doctor of Medicine at Yale University School of Medicine.

on the calcium concentration. The purpose of the present paper is to evaluate the relative effects of phosphorus and protein on serum calcium and to offer standards for the analysis of the causes of abnormal serum calcium concentrations.

Material and Methods.

It has been the custom in this laboratory, for some time, to analyze serum for inorganic phosphate and protein as well as calcium whenever determination of the latter was required. As a result of this custom a large number of simultaneous determinations of all three components have been made. Proteins were determined by a macro-Kjeldahl technique, calcium by the method of Kramer and Tisdall (12), phosphorus at first by Briggs' (13) modification of the method of Bell and Doisy, later by the procedure of Benedict and Theis (14). All blood was withdrawn without stasis and treated anaerobically by the methods described by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (15) until the serum had been separated. These precautions were observed to prevent exchanges of fluid between cells and serum as a result of altering the CO_2 and oxygen tensions of the blood. No anticoagulants were added to the blood samples. Serum was separated by centrifugalization and removed from the cells as soon as possible after the blood had been withdrawn.

TABLE I.

Nature of Clinical Material Studied.

Disease.	No. of patients.	No. of observations.
Chronic nephritis with uremia	11	29
" " " edema (nephrosis)	7	23
Acute nephritis	2	2
Fatal poisoning with bichloride of mercury	1	1
Pregnancy toxemia (nephrosis type)	1	1
Heart failure	2	2
Diabetes	12	4
Cholelithiasis with obstruction of common duct	1	1
Intestinal obstruction	1	1
Pyloric obstruction	2	1

In all, 73 observations were made on thirty-one patients with

diseases which are not generally believed to have any direct effect on calcium metabolism. The nature of these cases is shown in Table I. In this series serum inorganic P varied from 1.5 to 29 mg. per 100 cc., protein from 2.8 to 8.4 per cent.

Salvesen and Linder (3) have published forty-five observations on patients with cardiac and renal disease in which the same three components were simultaneously determined by methods similar to those employed by the authors. Salvesen and Linder also analyzed edema fluid and pleural and peritoneal transudates from some of their patients. The addition of their data to our own permits the extension of the range of variation of protein to lower levels. Some of the edema fluids were almost entirely free from protein.

On the basis of the authors' data and those of Salvesen and Linder (3), 118 observations in all, an attempt has been made to evaluate statistically the relative effects of inorganic phosphorus and protein on serum calcium. An alignment chart (Fig. 3) has been developed as a result of this analysis. It is intended to permit the prediction of the calcium concentration of the serum as far as this is affected by alterations of inorganic phosphorus and protein only. If the observed serum calcium diverges far from the predicted value it may be assumed that it is influenced by some other factor. If, on the other hand, predicted and calculated values coincide it may be inferred that there is no disturbance of calcium metabolism *per se*.

Construction of Chart.

Fig. 3 was constructed on the following principles. The effect of protein alone on calcium was first determined by an analysis of all those observations in which P was less than 5 mg. per 100 cc. The results of this analysis are shown in Table II and in Fig. 1. The determinations were divided into groups according to the serum protein level and average protein, phosphorus, and calcium concentrations in each group were estimated. There is a sufficient uniformity in the phosphorus values of the different groups to permit one to treat the group averages as if P were constant. In Fig. 1 these group averages are represented by crosses. There is nothing in the position of the points to indicate any significant curvature in either direction. This agrees with the reasonable

assumption that the extent of Ca-protein combination will bear a linear relation to the amounts of Ca and protein present. The best straight line drawn through the points is defined by the equation

$$\text{(1)} \qquad \text{Ca} = 0.556 \text{ protein} + 6$$

Hastings, Murray, and Sendroy (6), using certain data of their own and some selected from the observations of Salvesen and

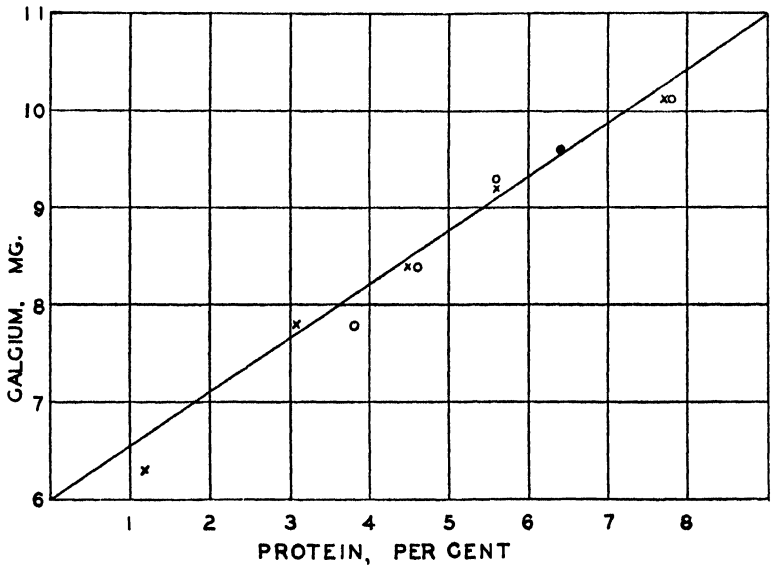

FIG. 1. The heavy diagonal line represents the mean curve of variation of calcium with protein, derived from all observations in which phosphorus was below 5 mg. per 100 cc. The crosses represent group averages from the combined data of the authors and Salvesen and Linder; the circles group averages from the data of the authors only.

Linder, obtained a straight line relation with the equation, Ca = 0.56 protein + 5.6.[1]

[1] The equation as actually given by Hastings, Murray, and Sendroy is Ca = 0.014 P − 1.4. The minus sign is evidently a typographical error. Calcium and protein are expressed in terms of millimols and gm. per kilo of serum water respectively, instead of the terms that we have employed.

The circles on Fig. 1 indicate the positions of group averages from the authors' observations only. It is evident that they

TABLE II.

Variation of Calcium with Changes of Protein When P < 5 Mg. per 100 Cc.

No. of observations.	Protein, per cent.		Average P.	Average Ca.
	Limits.	Average.		
			mg. per 100 cc.	*mg. per 100 cc.*
5	0–2	1.2	3.5	6.3
8	2–4	3.1	4.0	7.8
10	4–5	4.5	4.2	8.4
13	5–6	5.6	3.7	9.2
16	6–7	6.4	3.5	9.6
10	7–8.4	7.7	4.0	10.1

TABLE III.

Variations of Calcium with Changes of Phosphorus at Different Protein Concentrations.

No. of observations.	Average protein.	P, mg. per 100 cc.		Average Ca.
		Limits.	Average.	
Protein > 6 per cent.				
	per cent			*mg. per 100 cc.*
26	6.9	0–5	3.7	9.8
13	6.7	5–8	6.8	9.3
12	6.5	8–29	15.6	7.1
Protein 4 to 6 per cent.				
22	5.1	0–5	3.9	8.9
18	4.8	5–8	6.1	8.0
9	5.4	8–16	11.6	6.6
Protein < 4 per cent (sera only).				
5	3.5	0–5	4.3	8.0
5	3.5	5–8	5.8	6.9
2	3.8	{11.7, 18.9}	15.3	6.0

agree excellently with those obtained from the combined data. The addition of observations from Salvesen and Linder merely

permits more certain extension of the line to low protein limits. In general it may be stated that the two sets of data agree admirably.

Fig. 2 shows the relation of calcium to phosphorus at different protein concentrations. For its construction the system of group averages was again employed. The data used are presented in Table III. The crosses on Fig. 2 indicate the group averages with protein > 6, approximately defining the contour line, protein

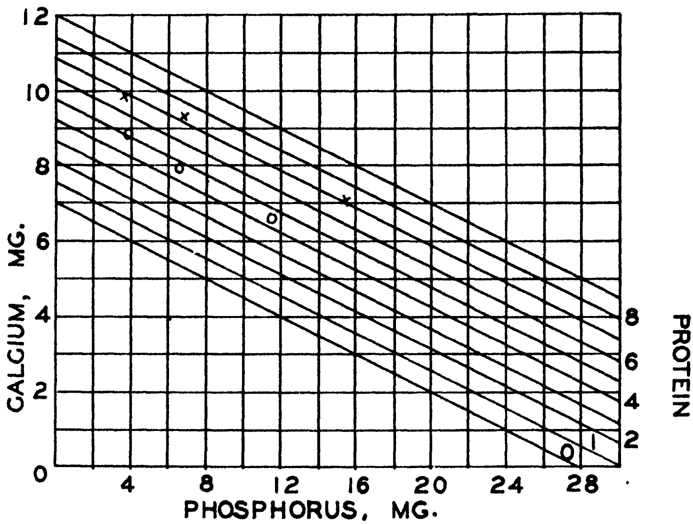

FIG. 2. The chart is drawn to conform to the equation Ca = −0.255 P + 0.556 protein + 7. The crosses represent group averages from observations in which protein was greater than 6 per cent. The actual protein concentrations, reading from left to right are 6.9, 6.7, and 6.5. The circles represent group averages from observations in which protein lay between 4 and 6 per cent. The actual protein concentrations, reading from left to right, are 5.1, 4.8, and 5.4.

= 6.8. The circles indicate the group averages with protein between 4 and 6 and similarly define the contour line, protein = 5.0. The general arrangement of the points in both series does not permit the deduction that the relations they represent are other than linear. The estimated slopes of the two best straight lines drawn through the two sets of points differ slightly. That of the 6.8 line is about −0.20, while that of the 5.0 line is −0.31.

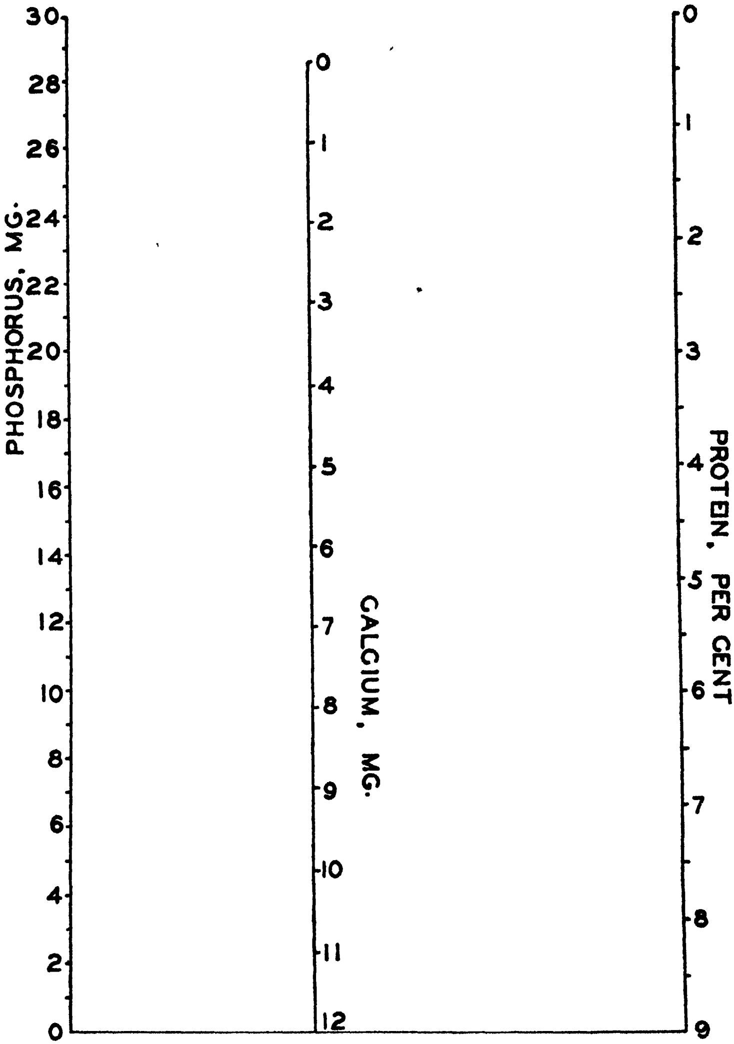

FIG. 3. The alignment chart is constructed to conform to the equation, Ca = -0.255 P + 0.556 protein + 7. To estimate the value of Ca to be expected with observed values of P and protein a line is drawn connecting the observed P and protein values and the concentration of Ca read where this line crosses the center ordinate.

As there is no reason to believe that the effect of phosphorus differs quantitatively at different protein concentrations and as both series contain approximately equal numbers of observations, it has been assumed that the protein contour lines are parallel, with a slope of -0.255, the average of the slopes of the 6.8 and 5.0 lines.

The relation of phosphorus to calcium can, therefore, be described by the equation

$$\text{Ca} = -0.255\ \text{P} + b \tag{2}$$

in which b is a variable determined by the intersection of the protein contour lines with the Ca axis; *i.e.*, when P = 0. The position of these lines when P = 4 mg. has been established by Equation 1. It is, therefore, a simple matter to establish their intersections with the Ca axis. When this is done it is found that

$$b = 0.556\ \text{protein} + 7 \tag{3}$$

Combining Equations 2 and 3, one obtains

$$\text{Ca} = -0.255\ \text{P} + 0.556\ \text{protein} + 7 \tag{4}$$

an equation which permits the prediction of Ca from P and protein. The alignment chart (Fig. 3) is constructed to conform with this equation.

DISCUSSION.

When Equation 4 is applied to the observed data it is found that Ca can be calculated with an average error of about ± 0.7 mg. per 100 cc. In eight instances the discrepancy between calculated and observed values exceeded 2.0 mg. per 100 cc. That the agreement should not be better is not surprising when one considers the great variability of the material investigated. In general the greatest discrepancies were observed in uremic cases in which P was extremely high. As patients in this condition almost always exhibit profound disturbances in the pH of the serum and in other electrolytes than those which have been here considered it is not surprising that this should be the case. Hastings, Murray, and Sendroy (6), Sendroy and Hastings (16), and Loeb and Nichols (8–10) have shown that the solubility of Ca in serum depends to some extent on both pH and total ionic strength.

The quantitative effect of these factors in serum has not yet been accurately established and our data are not sufficiently extensive to permit their evaluation. It is reasonable to believe that sufficient observations have been employed for the present analysis to obliterate for the most part the distorting influences of these inestimable factors.

Much more significant is the fact that both phosphate and proteins have an indubitable influence upon serum calcium. The obvious inference is that the physiological or pathological meaning of abnormalities of serum calcium cannot be evaluated

TABLE IV.

Variations of Ca in Relation to P and Protein in Two Cases.

Case No.	Date.	Serum protein.	P	Ca observed. (a)	Ca calculated (b)	Difference (a)-(b)
		per cent	*mg per 100 cc.*	*mg per 100 cc.*	*mg per 100 cc.*	*0.1 mg. per 100 cc.*
MD		8 4	4 4	9 8	10 5	+7
		5 9	2 9	8 8	9 5	+7
34640	Oct. 18, 1924	6 1	4 6	7 0	9 2	+22
	Nov. 7, "	5 8	4.3	8 6	9 1	+5
	Dec. 13, 1927	6 6	4 8	7 8	9 4	+16
	Jan 3, 1928	6 0	4 3	8 0	9 2	+12
	Mar. 21, "	6 0	4 1	8 6	9 3	+7
	Oct. 18, "	6 6	4 6	6 0	9 5	+35
	Nov. 7, "	6 5	5 0	7 0	9 3	+23
	" 23, "	6 1	5 3	8 4	9 0	+6

unless inorganic phosphate and protein are determined simultaneously, and then only by the aid of some standard for which the chart here presented offers a first approximation.

The value of such a standard is, perhaps, best illustrated by clinical examples. It has been demonstrated that insulin lowers the serum inorganic phosphate of patients with severe diabetes and hyperglycemia. At the same time calcium may change. Whether this change is due to demobilization of Ca to form some phosphate compound or is simply secondary to alterations of protein and P, has not been determined. Some light may be thrown on the question by observations on a case of diabetes.

The analytical data from this case, MD, are given in Table IV. At the time of the first observation the patient had acidosis, with the usual dehydration and extreme hyperglycemia. Calcium was also high. Although Ca fell with recovery the change was only of the degree that would be expected as a result of the reduction of proteins that occurred as the state of dehydration was overcome. This is indicated by the fact that the difference between observed and calculated values of Ca on the two occasions were identical.

Case 34640, Table IV, had a bilateral infection of the kidneys. She also had fractures of both femoral necks and some rarefaction of almost all the bones of the skeleton. Whatever may have been the underlying etiological factors, it was evident that there was a disturbance of the calcium metabolism that interfered with proper calcification of the bones. On three successive hospital admissions (the last because of a fracture of the right scapula), her calcium was low in proportion to protein and phosphorus. Each time, under treatment, including heliotherapy and cod liver oil (and, on the last occasion, calcium lactate) the discrepancy between observed and calculated serum calcium values diminished, calcium rising without a comparable change in protein and phosphorus. It is not improbable that in other cases of kidney disease, when calcium is found extremely low without important protein or phosphorus abnormalities, the calcium reductions are due to disturbances of calcium metabolism and the mechanism of calcification and not to the renal disease itself. Such hypocalcemias are usually encountered in children who are more prone to such disorders of calcification than adults.

The authors appreciate that on theoretical grounds the nomographic chart which has been developed is open to serious criticism as a description of the true influence of protein and phosphorus. It is to be expected that the effect of protein will be linear at constant pH and the number of observations employed is evidently large enough to smooth out the effects of pH variations on individual points. Probably the equation relating Ca to serum protein is a fairly exact statement of the calcium bound to proteins at the average pH of venous blood serum.

On the other hand, there is no theoretical reason to believe that the relation of Ca to P will be a straight line, if it is dependent upon

solubility products. Indeed Ca should not be directly related to P at all, but to some complex function of P which would be affected by pH. Professor A. B. Hastings, to whom this paper was submitted, suggested that, if pH determinations were available, an attempt be made to study the correlation of the calcium not combined with protein, the presumably ionized fraction, with the concentration of HPO_4 ions in the serum. If the solubility product of $CaHPO_4$ were the factor responsible for the effect of P on Ca, then the curve of ionized Ca and HPO_4 should be a right angle hyperbola and the curve relating their logarithms should be a 45° straight line.

Unfortunately pH had been determined in too few instances to permit an exact correlation of these functions. However, CO_2, which is a function of pH, had been determined in 65 instances. This was used to estimate HPO_4 indirectly. The results were not satisfactory. A logarithmic chart which was constructed showed great scattering and the best line made an angle far from 45° with the axes. This does not prove that the solubility product of $CaHPO_4$ is not the factor responsible for the effect of P on serum Ca. It may only mean that the calculations involved in the indirect estimation of HPO_4 were not sufficiently accurate.

It is not inconceivable that the statistical relation of the functions P and Ca in a large number of sera might prove to be linear as the resultant of two or more variables. In any case, the chart developed permits prediction of serum Ca with reasonable accuracy from P and protein. Undoubtedly the recognition of other determinants and the evaluation of their effects is of great theoretical and practical importance; but it is doubtful whether it will prove practicable to introduce more analyses as part of the routine procedure of calcium estimation.

SUMMARY.

1. The concentration of calcium in the serum varies directly with the concentration of protein and inversely with the concentration of inorganic phosphorus. It is, therefore, impossible to interpret serum calcium variations accurately unless protein and inorganic phosphorus are simultaneously determined.

2. 118 simultaneous observations of serum Ca, inorganic P, and protein from this laboratory and from a previous report of

Salvesen and Linder have been analyzed to evaluate the relative effects of protein and phosphorus on serum calcium. The sera were obtained from patients, chiefly with nephritis, in whom there was no reason to suspect the presence of a true defect in calcium metabolism.

3. The relation of the three components in this series is defined by the equation, Ca = −0.255 P + 0.566 protein + 7. An alignment chart has been constructed to conform with this equation.

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THE PRODUCTION OF EDEMA AND SERUM PROTEIN DEFICIENCY IN WHITE RATS BY LOW PROTEIN DIETS.

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This study was undertaken in an effort to learn something of the mechanism of the production of malnutrition edema. It was decided to attempt to repeat the work of Kohman (1) who produced the condition experimentally by feeding low protein diets, and to study the effect of such diets on the serum proteins.

In 1895 Starling (2) first showed that the colloid osmotic pressure of the serum was proportional to the concentration of protein in it. He advanced the theory that decreased osmotic pressure in the serum was responsible for the deposition of water in the tissues. Disagreeing with Starling, Martin Fischer (3) claimed that edema resulted from alteration of the hydrogen ion concentration of blood and tissues which influenced the imbibition of fluid by the tissue proteins. Subsequent work has not supported Fischer's theory, but has increasingly substantiated that of Starling.

Govaerts (4) measured the colloid osmotic pressure of the serum of individuals, normal and otherwise, and found that an osmotic pressure lower than a certain figure was associated with a tendency toward edema (5). This lowered pressure could be caused by reduction in the total amount of protein, but especially by a loss of serum albumin (6). If the capillary pressure exceeded the osmotic pressure of proteins, water was deposited in the tissues. This held for edemas caused by stasis as well as others. Schade and Claussen (7) confirmed Govaert's work in all respects by their "onkometer" methods. In this country, Epstein (8) found lowered serum proteins in certain cases of renal disease, especially the type called nephro-

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* The data in this paper are taken from the thesis of Dr. R. A. Frisch done in partial fulfilment of the requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

sis, and suggested that, in order to relieve the edema, the serum protein be raised by increasing the protein intake. Peters (9) has emphasized the prevention of malnutrition in patients with renal disease by adequate dietary protein. He found (10) that in cases of malnutrition of any kind, the serum proteins were lowered and there was a tendency toward water retention.

During and just after the late war, edema was noticed in Central Europe where people were forced to subsist largely on turnips and other root vegetables for long periods of time. Schittenhelm and Schlecht (11) studied the condition in prison camps and decided that rest and increased protein and fat in the diet relieved the edema. They estimated serum proteins by refractometer methods and found that refractometric indices were low in 50 per cent of the cases. Finally the disease was attributed to a lack of protein and possibly fat in the diet. The symptoms were not those of a lack of vitamins. Jansen (12) found the condition similar to cachectic edemas reported by Govaerts in the reduction of serum protein, particularly of the albumin fraction.

In 1918 Denton and Kohman (13), while working on the dietary properties of carrots, noticed that some of their animals, which were fed on little else over extended periods, developed edema. In later work both Maver (14) and Kohman (1) found that a certain number of white rats developed edema when fed diets containing carrots, cornstarch, lard, and salts for periods of 8 to 12 weeks. Various food elements were added to the diets in an effort to bring them back to normal; but nothing succeeded that did not contain protein. Kohman (1) thought that the symptoms produced might be related to the famine edema of the war. Her diets eliminated from consideration every factor except the protein and she attributed the condition to a deficiency of that element. This work seemed worthy of repetition and further investigation. It was decided, first, if possible, to produce edema by the carrot diets and then learn a little more of the mechanism of its production by a study of the serum proteins.

EXPERIMENTAL.

The two diets whose composition is shown in Table I were chosen. They were the same except for the fact that in the second the carrots were dried at low temperature while, in the first, they were merely ground up fresh and mixed. The amounts of carrots fed gave an ample supply of vitamins. Kohman at first questioned the supply of vitamin B, but found that the addition to

the diet of wheat germ did not alter the experimental results. She could detect no difference when the salt content was varied. The only source of protein is that contained in the carrots.

Young, growing albino rats varying in weight from 50 to 70 gm., obtained from the Yale Department of Physiological Chemistry and Connecticut Agricultural Experiment Station strains, were used.

Blood samples for serum protein determinations were drawn by heart puncture without exposure to air (15). Because of the size of the animals it was impossible to get sufficient blood to determine protein fractions nor could the total amount of serum protein be corrected for the non-protein nitrogen. 0.5 to 1 cc. was the usual

TABLE I.

Composition of Diets.

	Wet diet.	Dry diet.
	gm.	*gm.*
Carrots	4500	550
Starch	360	360
Lard	60	60
Salt mixture*	35 6	35 6
	per cent	*per cent*
Proteins	0 7	6 3
Fats	1.6	7.0
Carbohydrates	15.5	68 8
Salts	1.4	6 3
Water	80 0	13 0

* McCollum, E. V., and Davis, M., *J. Biol. Chem.*, **21**, 615 (1915).

amount of blood obtained and special tubes were made for centrifuging it. The gasometric micro-Kjeldahl method of Van Slyke (16) was used after it had been found to check excellently with the usual macro-Kjeldahl method. All determinations were made in duplicate on the diluted serum. The omission of non-protein nitrogen determinations cannot alter the deductions which have been made from the analytical data. The total amount of such nitrogen in normal rat blood is so small that its complete elimination from the blood as the result of the experimental procedure would have no important influence upon the reported serum protein changes.

In the first group of animals only those on the dry diet were given extra water. The animals on the wet diet could not eat enough to keep up to their normal caloric level and maintained a lower weight than the others, although their growth curves (Fig. 1) are essentially the same in character. At first there is a rapid decline in weight, no doubt due in large measure to adjust-

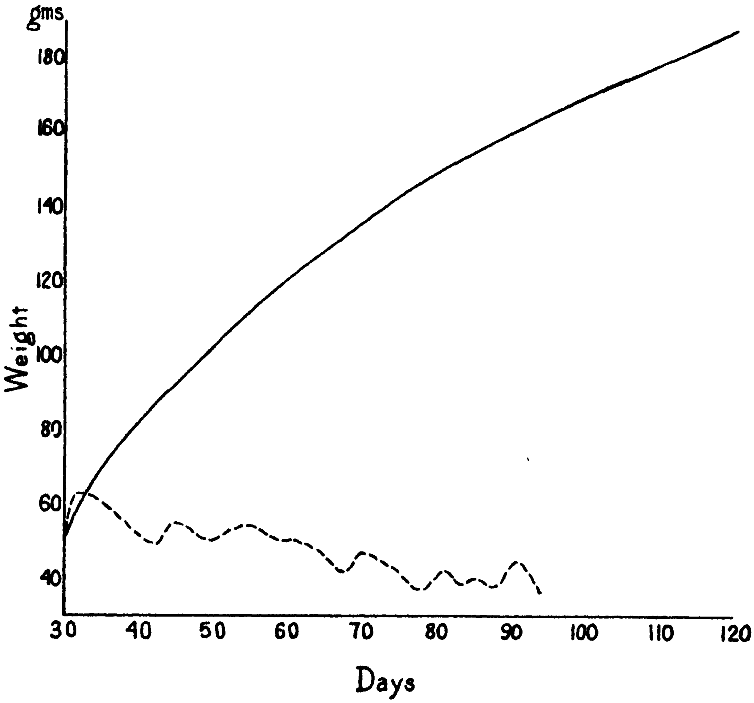

FIG. 1. Composite growth curve of animals on the wet diet. Solid line = wet diet with casein (five rats); broken line = wet diet without casein (fourteen rats).

ment to the diet; later there may be a slight recovery followed by a slow decline until the animal reaches a level at which it stays until a week or two before death, when there are fluctuations, more marked in some cases than others. Generally the life of a rat on the dry diet was longer than that of one on the wet. Toward the end the animals became very emaciated and their hair lost its

smooth appearance. They presented no eye symptoms nor paralyses. Their deaths as observed in two cases were not marked by convulsions nor signs of spasticity.

Edema, unless considerable, was difficult to observe in the living animal. It was variable from day to day and slight puffiness about the face and neck could not be easily detected. The condition was usually most noticeable in the dependent parts; namely,

TABLE II.

Relation of Diet to Production of Edema and Serum Protein Deficiency.

Group No.	Diet.	No. of animals.	Average calories per day.	No. of animals that died.*	No. of animals that developed edema. Total.	No. of animals that developed edema. Demonstrable during life.	No. of serum protein determinations.	Average serum protein, per cent
I	Wet diet without extra water.	6	20	3 Ex 1 RI 2 HS	2	1	2	3.2
II	Wet diet with extra water.	14	22	9 Ex 3 RI 2 HS	8	4	6	3.8
III	Wet diet with protein	5	45	0	0	0	5	6.9
IV	Dry diet without extra protein.	5	40	1 Ex 1 RI 1 HS	4	3	2	3.2

* Ex, died as a direct result of the experimental procedure. RI, died as a direct result of respiratory infection. HS, died as a direct result of heart puncture.

the anterior thoracic and abdominal walls. Sometimes when the skin was pricked with a needle, a drop of fluid would exude. Upon autopsy, general anasarca of varying degree was the most striking phenomenon. There was practically no gross fat visible and the muscles were small and pale. No other abnormalities were noted except in animals which died as a result of infection, when lung changes were observed.

The experimental results are epitomized in Table II.

Groups I and II in Table II show the result of the wet diet. Kohman did not supply extra water because of the amount in the diet, but in these investigations it was decided to allow the animals, except those of one group, to drink as much as they wished, as did those on the dry diet. However, they took very small

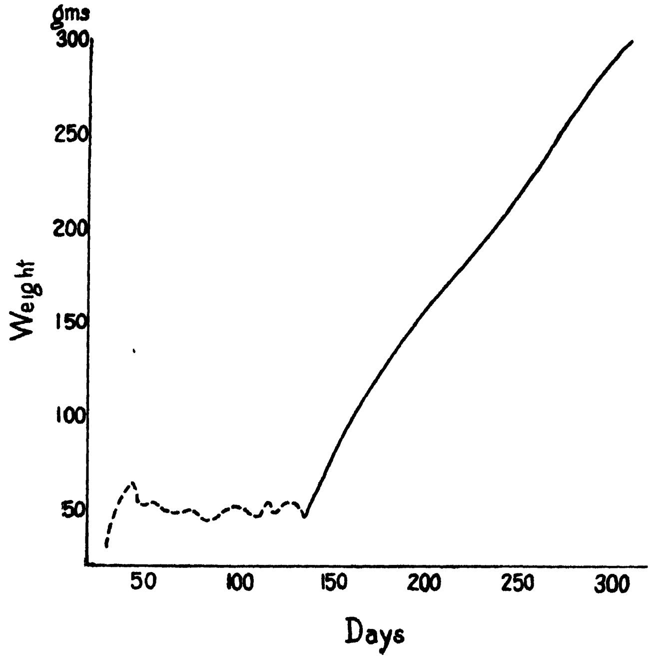

FIG. 2. Composite growth curve of animals on the dry diet. Broken line = twelve animals on dry diet; solid line = two of the twelve animals after the addition of protein to the diet

amounts. Of six animals of Group I without extra water, edema appeared after about 10 weeks on the diet in two, one of whose serum proteins were 3.0 per cent. Those of one which showed no edema, but merely extreme emaciation, amounted to 3.4 per cent. Of Group II of fourteen animals, eight showed water in the tissues after 8 to 14 weeks, three of them to a most extreme degree. The average of six protein determinations was 3.8 per cent. The serum

protein content of those animals which did not develop edema was uniformly slightly higher than the proteins of those which did. Just before the animals were put on the experimental diet, blood samples for serum protein determinations were drawn from six of the same group of approximately the same weight and age. The average of the serum proteins of these six normal animals was 5.5

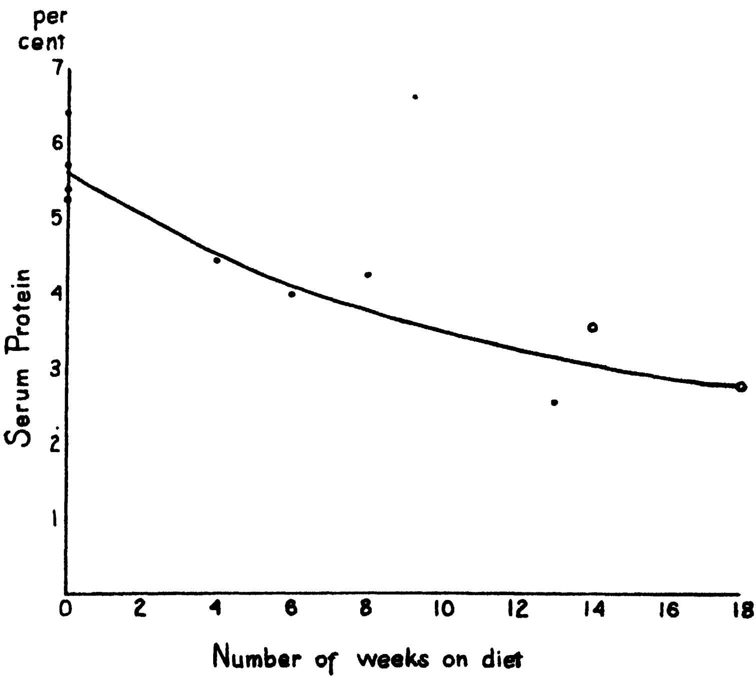

Fig. 3. Curve illustrating the fall of serum proteins on the dry diet. The solid points = individual observations; the circles = animals which developed edema.

per cent. The average of five adult rats taken at random on adequate diets was about 8.0 per cent.

To determine the adequacy of the wet diet with respect to other factors than protein, five animals were put on a diet of the same materials and composition except that the starch up to 18 per cent of the total calories was replaced by vitamin-free casein (Harris). These animals presented a marked contrast to those of the previous

groups. They continued to gain in weight at first as rapidly as those on a standard diet, later somewhat more slowly. Their appearance was always healthy, they showed no edema, and ate an increasing instead of decreasing amount of food. Fig. 2 is a composite growth curve of the group. Blood samples for serum protein determinations were drawn after 11 to 12 weeks on the diet. The results of these are shown in Table II, Group III.

The serum proteins were very slightly lower than those of the five normal adults, but considerably higher than those of the six young animals before the start of the experimental period.

Table II, Group IV, shows the results of animals on the dry diet. They were allowed to drink as they wished. The only animal of this group which did not show edema died at about 7 weeks of age as a result of a lung infection. The others all showed definite signs after 9 to 14 weeks on the diet. Serum protein determinations made on two animals averaged 3.2 per cent. The two survivors, one male and one female, one of which had blood drawn for analysis, were then put on a diet the same in every respect as before except that the starch up to 18 per cent of the total calories was replaced by vitamin-free casein (Harris). They lost their edema, gained weight rapidly, and were mated after 2 months, when the male weighed about 300 and the female 200 gm. A litter of eleven resulted, all of which lived and gained well until after weaning, when they were discarded.

In another group of animals the curve of the serum proteins throughout the experimental period was studied. Fig. 3 shows the results. The figures for the six normal animals determined previously were taken as a standard and samples drawn at intervals. There was a gradual decline in the amount of serum protein. The last three animals showed some degree of edema while living and post mortem, and all of these had serum protein values lower than 4.0 per cent.

DISCUSSION.

The number of animals used was too small to permit deduction as to the frequency with which edema will develop on these diets, but there is no question that it does appear as a direct result of the diets. In Kohman's work the animals on the wet diet showed edema more frequently than did those on the dry, while in this

investigation the relation seems to be reversed. However, larger groups of animals would give a better comparison of the two diets. Those on the dry diet certainly ate more food and more protein calories. A better method of determining the degree of water retention than gross examination might give better figures for comparison as well as clarify some of the apparently negative results. Nevertheless an edema has been produced in healthy animals as the direct result of a nutritional deficiency.

Everything seems to point to lack of protein as the nutritional defect responsible for this condition. The animals on the wet diet ate less than 0.04 gm. of protein per gm. of rat per day and those on the dry diet less than 0.12 gm. This is definitely less than their protein requirement. Kohman could find no other factor responsible. She tried diets with greater amounts of fat, greater

TABLE III.

Concentration of Proteins in Serum of Rats.

	Normal adult rats	Rats on wet diet with added casein.	Normal rats 1 month old.	Rats on wet diet	Rats on dry diet.
No of determinations	5	5	6	8	5
Average serum proteins, *per cent*...	8 0	6 9	5.5	3 6	3 0

and smaller amounts of salt, and extra vitamins, but her results remained unchanged until protein was added. Finally, with a synthetic diet complete in every respect except for protein, she produced edema in a small number of animals. In our studies the addition to the wet diet of casein alone caused the animals to run an entirely different course. This and the fact that two animals which had developed edema on the dry diet, after the mere addition of casein grew and reproduced normally, point strongly to the protein deficiency as the cause of the edema.

The determinations of serum proteins show a marked reduction in almost every case after a period of 2 to 3 months on the carrot diet. This fact is in keeping with previous experimental work on the relation of edema and serum protein deficiency. The serum proteins of these rats, in general, were lowered about 40 per cent from the figure at the start of the experimental period. However,

young rats seem to have less serum protein than the adult, a fact which indicates that the actual reduction is greater. Table III shows the average serum protein values of each of the groups studied.

There has not been a sufficient number of determinations to detect variations between the sexes, but there has been no indication of any great difference. Both sexes were used indiscriminately. The observed reduction in the serum proteins has been the direct result of low protein feeding. A similar reduction might occur with other dietary deficiencies, such as some vitamin lack. Certainly, where the problem has been studied, serum proteins have been found lowered in humans with malnutrition from any cause, but no report of any reduction produced experimentally has been published until the work of Leiter (17). He bled dogs, centrifuged off the plasma, mixed the cells with normal saline, and replaced them in the animals. By this means the serum proteins were lowered and an edema developed. His work has been confirmed by Darrow and Hopper (18) in the Yale Department of Pediatrics. In infants with digestive disorders, who were being fed on diets of flour or rice and water and developed the condition called *Mehlnahrschaden* by Czerny, Gorter (19) noticed edema along with the other symptoms. If serum proteins had been determined they probably would have proved to be low as a result of the protein starvation, as was found in the famine edemas. These infants improved rapidly with added protein in the food and blood transfusion. Neither butter, salt, nor sugar added to the food relieved the condition.

The serum proteins are, of course, only partly responsible for the production of edema. Here, as in other conditions, they determine a tendency toward water retention which is influenced by other factors. The fluid intake is very important, for if it is reduced in any way, voluntarily or by some other means such as vomiting, no edema develops, even if the serum proteins are actually quite low. In the experiments described fluids were neither forced nor restricted, but the animals were allowed to drink as they wished. The rôle of salt has been studied a great deal, but its importance is not yet clear. More complete studies of the nitrogen metabolism, particularly the nitrogen equilibrium, in these animals might clarify the picture. Blood electrolyte

studies would help if they could be made. The basal metabolic rate probably would be found lowered as in malnourished humans. However, since Starling's first work, the lowering of the serum proteins has assumed increasing importance and appears to be the factor most concerned in the production of edema not caused by mechanical stasis.

SUMMARY.

A study has been made of the edema produced by Kohman in white rats by means of low protein diets. Kohman's work has been confirmed. Serum proteins were determined in normal rats and in rats on the experimental diets. They were found to be reduced in animals on the low protein diets, whether or not edema developed.

The authors wish to take this opportunity to express deep appreciation for the aid and advice of Dr. Anna J. Eisenman in the blood analyses.

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NOTE ON THE DISSOCIATION CONSTANTS OF CERTAIN AMINO ACIDS, INCLUDING VALINE, GLUTAMIC ACID.

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In a series of papers (1–3) published by the present writer in 1923 the dissociation constants of amino acids were determined from titration curves. Among these amino acids were glutamic acid and valine. Kirk and Schmidt (4) have recently (1929) referred to the main paper (1) where only a general summary of the conclusions regarding glutamic acid and valine was given, but unfortunately have entirely overlooked the two subsidiary papers (2, 3) in which the actual curves and fuller values for these two amino acids were set out. This led them to the following erroneous conclusions.

" . . . the dissociation constants for glutamic acid were determined by somewhat inadequate methods [Holmberg, 1908; Walden, 1891]. Harris [1923] has published the dissociation constants for valine, but without titration curves, and with an omission in the tables, making it desirable to repeat the determination of the constants for this amino acid."

It now becomes of interest to compare the values newly obtained by Kirk and Schmidt with those previously determined by the writer (see Table I).

The agreement between the two sets of readings is very satisfactory, and indeed amounts to virtual identity, for some variation in the second decimal place falls within the experimental error, with the technique employed. The experimental conditions chosen by Kirk and Schmidt are the same as those employed by myself ($t = 25°$; dilution about 0.05 M; quinhydrone electrode used in acid solutions) and the device of introducing a blank correction for the solvent is also made, as described in my several

TABLE I.

Dissociation Constants for Glutamic Acid and Valine.

	Kirk and Schmidt (1929).	Harris (1923).
Glutamic acid.		
pk'_1 (*i.e.*, $14 - pk'_b$)*	2.19	2.11
pk'_2 (" pk'_{a_1})	4.25	4.21
pk'_3 (" pk'_{a_2})	9.66	9.79
Valine.		
pk'_1 (*i.e.*, $14 - pk'_b$)	2.32	2.30
pk'_2 (" pk'_a)	9.62	9.70

* k values have been here converted by the writer into pk units for the sake of easy comparison.

papers. With regard to the question of the activity coefficients of amino acids, reference may be made to a note published in 1925 (5).

It was mentioned by the writer in 1923 that he had reported on the titration dissociation constants of all the amino acids then known to occur in protein with the exception only of serine, hydroxyglutamic acid, and proline and oxyproline.[1] The proposed publication of these further values (delayed by several years' absence from academic work and by ill health) is now no longer necessary, since Kirk and Schmidt (4) and McCay and Schmidt (6), have now filled in these several gaps. The exact values for cystine worked out by Cannan and Knight, 1927, (7) should likewise prove of much value.[2]

[1] Similarly Kirk and Schmidt write (1929): "It has been previously pointed out [McCay and Schmidt, 1926] that values for the dissociation constants of oxyproline, β-hydroxyglutamic acid, and serine are still lacking."

[2] For cystine, as Professor Schmidt kindly draws my attention, Cannan and Knight's (1927) values are in moderate agreement also with those of Sano (1926) (8).

With regard to leucine, Harris (1923) (1) found pk′ values of 2.36 and 9.60. Similarly, in the course of an independent study, conducted along identical lines, Hirsch (1924) (9) quoted values of 2.27 and 9.74 for "isoleucine." Finally Kirk and Schmidt, possessing no knowledge of Hirsch's

SUMMARY.

Kirk and Schmidt's values for the titration dissociation constants of valine and glutamic acid (1929) are virtually identical with those determined by the author (1923).

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values, now obtain results in good agreement (1929) (4); *viz.*, 2.36 and 9.68 for isoleucine.

For arginine certain anomalous values have recently been published: a communication is in preparation on the question.

STROPHANTHIN.

XVII. DEHYDRATION AND LACTONE CLEAVAGE IN ISOSTROPHANTHIC ACID DERIVATIVES.

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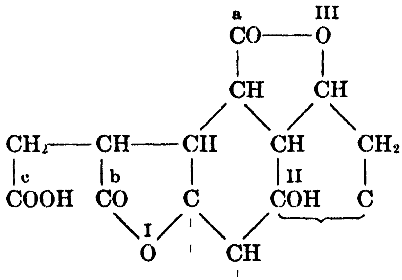
(Received for publication, July 1, 1929.)

Of considerable importance for the further elucidation of the structure of strophanthidin has been the study of the lactonization of the previously described dibasic keto acid, duodephanthondiacid.[1] The results of this study will be presented more fully in a subsequent communication. In the course of this work a number of methods have been employed to accomplish the desired lactonization to the unsaturated lactone. Among these was the use of acetic anhydride containing a small amount of acetyl chloride. When the acid was heated in this mixture, a crystalline reaction product was obtained but of rather unexpected chemical character. In addition to the desired lactonization, it was found that other portions of the molecule participated in the reaction. As an aid to the proper interpretation of the nature of the side reactions it was found useful to study the effect of the reagent on an isostrophanthic acid derivative in which the possibility of the above type of lactonization was excluded.

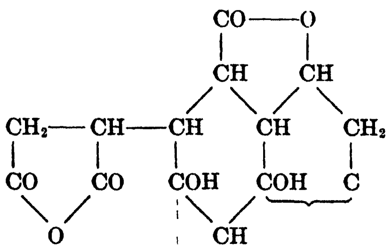
For this purpose β-isostrophanthic lactone acid[2] was chosen as a convenient object. As shown in Formula I given below, carboxyl (a) forms with hydroxyl III a very stable lactone group so that participation in the reaction by these two groups appeared unlikely. From this lactone acid a crystalline substance was obtained of similar general character to that obtained from duodephanthondiacid. It proved to be neutral. The formula $C_{25}H_{30}O_7$ was derived from the analytical figures. Although

[1] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **79**, 548 (1928).
[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 833 (1927).

neutral, the behavior of the substance towards alkali at once disclosed its acid anhydride nature. On saponification with 0.1 N alkali, a total of 3 equivalents was consumed, the extra 1 of which was found to be required by an acetyl group. These observations were explained by the series of reactions shown in Formulæ I to III.



I.



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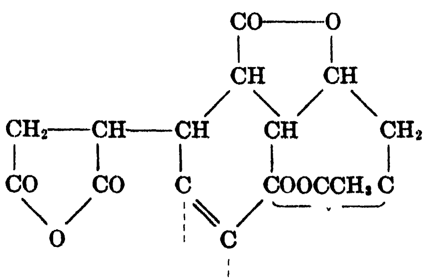

III.

The labile lactone group of carboxyl (b) on hydroxyl I (Formula I) was opened, with the formation of a substituted succinic anhydride (Formula II). Simultaneously, under the vigorous dehydrating conditions, hydroxyl I, which had now become exposed, was removed with the formation of a double bond (Formula III). In addition, hydroxyl II was acetylated during the reaction.

After saponification by 0.1 N alkali, the crystalline, unsaturated dibasic lactone acid was obtained in which the stable lactone group had remained unattacked. This acid was further characterized by its neutral dimethyl ester. The unsaturated nature of this series of substances was then shown by the production of a dihydro derivative by hydrogenation of the dimethyl ester.

Further study has shown that the reaction with acetic anhydride and acetyl chloride can occur only when the carboxyl (c) of isostrophanthic lactone acid is free. When the same reaction was attempted with the methyl ester, the latter was converted only into the acetate involving hydroxyl II. The condition of this reaction appeared, therefore, to be the fixing of the free carboxyl group in anhydride formation with the carboxyl originally belonging to the labile lactone group. In turn, a reversal of the reaction by relactonization on hydroxyl I was rendered impossible by the removal of the latter to form a double bond. It appeared possible that similar conditions could be realized by heating α-isostrophanthic lactone acid (or its methyl ester in this case) with dry methyl alcohol containing a small quantity of hydrogen chloride in which cleavage of the lactone group with formation of the dimethyl ester might occur with simultaneous removal of the exposed hydroxyl I.

This was substantiated by the ready formation of the neutral unsaturated dimethyl ester $C_{25}H_{34}O_7$. However, this ester was not identical but proved to be isomeric with the neutral dimethyl ester of the acid formed by the anhydride method. The difference was found to be due to the isomerizing effect of the free hydrogen chloride which caused the formation of a derivative of the δ-isostrophanthic acid series. This type of isomerization has already been discussed in the case of isostrophanthic acid itself, in which α-isostrophanthic acid may be isomerized to the γ form or the β-acid to the δ form.[3]

[3] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 831 (1927).

The conclusion as to the nature of the isomerism of these esters was based on the following considerations. From the specific rotations of the two isomeric esters the provisional assumption appeared justified that the levorotatory isomer obtained by the acetic anhydride method is still a β-isostrophanthic acid derivative and the dextro ester obtained by the use of methyl alcoholic hydrogen chloride is a δ derivative. An attempt was made to demonstrate this relationship directly by the conversion of the former into the latter by heating with methyl alcoholic hydrochloric acid. The levo isomer, however, was recovered unchanged. Since the original anhydride had been saponified to the succinic acid by the use of alkali, the possibility appeared that here the isomerization may have occurred. To test this possibility, the dextrorotatory isomer was then treated with alkali. It was, however, recovered unchanged after reesterification of the resulting acid. Therefore, alkali played no rôle in the observed isomerization. It appears, therefore, that hydroxyl I or the carbon atom which carries it is a necessary factor in this isomerization and that in the formation of the levo isomer by acetic anhydride-acetyl chloride removal of hydroxyl I occurred before opportunity for isomerization could take place. With methyl alcoholic hydrochloric acid the isomerization must occur before cleavage of the lactone group.

That the isomerizing effect of acid appears to involve the carbon atom bearing hydroxyl I, has already been the subject of discussion in the case of the transformation of strophanthidin into pseudostrophanthidin[4] and of strophanthidinic acid into its neutral dilactone[4] under the influence of strong acids.

Since by these reactions a means has been found to dispose of the lactone group and hydroxyl I of the isostrophanthic acid series, it was hoped that by the use of stronger methyl alcoholic hydrogen chloride solutions hydroxyl II might also be removed as in the case of the formation of the ethylal of dianhydrostrophanthidin.[5] The reaction, however, appeared to stop with removal of hydroxyl I. It would appear that in the formation of monoanhydrostrophanthidin the double bond is differently placed ($\Delta^{\beta,\gamma}$) to the carbon atom bearing hydroxyl II than in the unsaturated substances of the present report which may be $\Delta^{\alpha,\beta}$. These rela-

[4] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **74**, 798 (1927).

[5] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, **59**, 723 (1924).

tionships we hope to explain more fully when more data have been collected.

The methods used here for the cleavage of the lactone groups in the iso series have been applied to other derivatives of the isostrophanthic acids as well as to the iso acids of other cardiac aglucones. The results will be presented subsequently in their appropriate connections.

EXPERIMENTAL.

β-Isostrophanthic Lactone Acid with Acetic Anhydride and Acetyl Chloride.—0.5 gm. of β-isostrophanthic lactone acid was heated in a sealed tube with 9 cc. of acetic anhydride and 1 cc. of acetyl chloride, at 80°. The substance gradually dissolved and solution was complete within an hour. The heating was continued for 16 hours after which the acetic anhydride and acetyl chloride were removed by distillation under diminished pressure. The glassy residue was taken up in chloroform and the chloroform solution was washed successively with dilute sodium carbonate and water. Evaporation of the dried solution left a resin which readily crystallized under acetone. Recrystallized by careful dilution of its acetone solution it crystallized beautifully as stout needles which were neutral and solvent-free and melted at 280–281°. From acetone the substance separated as wedge-shaped plates. The yield of recrystallized material was 0.4 gm. It is readily soluble in chloroform, more sparingly so in acetone, and but sparingly soluble in alcohol.

That the substance is an acid anhydride was shown by titration experiments. 18.875 mg. were suspended in 2 cc. of alcohol and directly titrated in the cold with 0.1 N NaOH, with phenolphthalein as indicator. The alkali, which was very rapidly taken up at first, was later consumed more sluggishly, until 0.586 cc. of 0.1 N NaOH had been added, after which further direct titration of the anhydride group was impossible because of the slowness of the consumption of base. Accordingly, excess 0.1 N NaOH was added and the solution was refluxed for 3 hours in an atmosphere of nitrogen. By this means the total base consumed was increased to 1.329 cc. of 0.1 N NaOH. Calculated for 3 equivalents for $C_{25}H_{30}O_7$ is 1.280. The stable lactone group of the substance, in accord with previous experience with the parent lactone acid, was practically unattacked by this treatment.

4.185 mg. substance: 2.630 mg. H_2O, 10.390 mg. CO_2.
4.165 " " : 2.554 " " 10.352 " "

$C_{25}H_{30}O_7$. Calculated. C 67.84, H 6.84.
Found. " 67.71, " 7.03.
" " 67.78, " 6.86.

Saponification of the Anhydro Anhydride Acetate.—0.1 gm. of the previous anhydride acetate was suspended in 5 cc. of alcohol and treated with 20 cc. of 0.1 N NaOH. After heating on the steam bath for 3 hours, the mixture was acidified with acetic acid. The dibasic anhydro lactone acid slowly crystallized as leaflets which were collected with water. When it was air-dried, it melted at 230–232° with slight preliminary sintering and contained water of crystallization. Recrystallization from organic solvents proved impracticable owing to its great solubility, so that the acid was preferably converted into the dimethyl ester which proved to have excellent properties. For analysis the acid was dried at 100° and 15 mm.

3.785 mg substance: 0.097 mg. H_2O.
$C_{23}H_{30}O_7 \cdot \frac{1}{2}H_2O$. Calculated. H_2O 2.11.
Found. " 2.56.

4.232 mg. substance: 2.802 mg. H_2O, 10.210 mg. CO_2.
3.688 " " : 2.403 " " 8.879 " "

$C_{23}H_{30}O_7$. Calculated. C 65.99, H 7.23.
Found. " 65.79, " 7.41.
" " 65.66, " 7.29.

The β-Dimethyl Ester.—The previous acid was esterified in acetone solution with diazomethane. Evaporation of the solvent left a crystalline residue. When recrystallized from methyl alcohol, it formed needles which melted at 199–200° and were solvent-free. It is readily soluble in the alcohols, chloroform, and acetone. In the latter solution it is slowly attacked by permanganate. In sulfuric acid it dissolves with a yellow color which deepens slowly through orange and brown to a deep green, on long standing. When heated at 80° for 16 hours with a 1 per cent methyl alcoholic hydrogen chloride solution, the ester was recovered unchanged.

$[\alpha]_D^{29} = -28.0$ (c = 0.497 in methyl alcohol).

4.970 mg. substance: 3.460 mg. H_2O, 12.220 mg. CO_2.
3.070 " " : 2.150 " " 7.560 " "

$C_{25}H_{34}O_7$.	Calculated.	C 67.22, H 7.68.
	Found.	" 67.05, " 7.79.
	"	" 67.16, " 7.83.

The Anhydro Anhydride Acetate and Methyl Alcoholic Hydrogen Chloride.—When the anhydro anhydride acetate was subjected to treatment with methyl alcoholic HCl, the anhydride group was opened to form the neutral dimethyl ester but the acetate group remained unattacked.

0.09 gm. of the anhydride was heated in a sealed tube with 4 cc. of 1 per cent dry $CH_3OH \cdot HCl$ at 75–80°. The substance dissolved completely within 45 minutes. After 16 hours heating, a few drops of saturated sodium acetate solution were added and the mixture was concentrated to 1.5 cc. Careful dilution with water caused the separation of needles and blades. The collected substance was recrystallized from dilute methyl alcohol and formed needles which were anhydrous and melted at 170–171°.

4.142 mg. substance: 2.823 mg. H_2O, 10.100 mg. CO_2.

$C_{27}H_{36}O_8$.	Calculated.	C 66.35, H 7.43.
	Found.	" 66.50, " 7.63.

Hydrogenation of the Anhydro Ester.—The unsaturated dimethyl ester was slowly hydrogenated in methyl alcoholic solution with platinum oxide catalyst. The filtrate from the catalyst left on evaporation a residue of needles. Recrystallized by strongly cooling its methyl alcoholic solution, it separated as needles which were collected with cold methyl alcohol. The substance sintered at 219° and melted at 229–231°. Its solubilities resemble those of the unsaturated ester except that it is more sparingly soluble in the alcohols. With H_2SO_4 it gives at first a light yellow coloration, turning through golden orange to brown on standing. The substance is stable to $KMnO_4$ in acetone solution. For analysis the substance was dried at 100° and 15 mm.

3.430 mg. substance:	2.443 mg. H_2O,	8.400 mg. CO_2.	
3.710 " "	: 2.690 "	" 9.135 " "	
5.424 " "	: 5.580 "	AgI.	

$C_{25}H_{36}O_7$.	Calculated.	C 66.92, H 8.09, OCH_3 13.84.
	Found.	" 66.79, " 7.97.
	"	" 67.15, " 8.11.
	"	OCH_3 13.57.

β-Isostrophanthic Lactone Acid and Methyl Alcoholic Hydrogen Chloride (δ-Dimethyl Ester).—0.1 gm. of the lactone acid was heated in a sealed tube with 3 cc. of 1 per cent dry methyl alcoholic HCl at 75–85° for 17 hours. On careful dilution of the resulting solution the substance readily crystallized in the form of needles and blades and proved to be a neutral ester. It was recrystallized by careful dilution of its methyl alcoholic solution and separated again as long needles in anhydrous form which melted without decomposition at 154–155°. The yield was 0.09 gm.

$[\alpha]_D^{29} = +90$ (c = 0.480 in methyl alcohol).

4.460 mg. substance:	3.205 mg. H_2O, 10.990 mg. CO_2.		
5.550 " "	: 3.897 " " 13.645 " "		
4.704 " "	: 4.913 " AgI.		
$C_{25}H_{34}O_7$.	Calculated.	C 67.22, H 7.68,	OCH_3 13.90.
	Found.	" 67.20, " 8.02.	
	"	" 67.05, " 7.85.	
	"		OCH_3 13.78.

When in the above reaction the lactone acid was heated with 5 per cent dry methyl alcoholic HCl the same dimethyl ester was obtained in excellent yield. When the ester was saponified and the resulting acid extracted after acidification, reesterification by diazomethane resulted only in the recovery of δ-dimethyl ester. This shows that no isomerization accompanies saponification with alkali.

Acetate of β-Isostrophanthic Lactone Acid Methyl Ester.—0.1 gm. of the methyl ester of β-isostrophanthic lactone acid was heated with 1.8 cc. of acetic anhydride and 0.2 cc. of acetyl chloride at 75–85° for 15 hours. After removal of the excess reagent under reduced pressure the resulting resin was taken up in $CHCl_3$ and washed with dilute Na_2CO_3 and water. Evaporation of the dried solution gave the substance directly as a mass of radiating needles. Recrystallized by dilution of its acetone solution it formed needles which were anhydrous and melted at 235–237°.

4.355 mg. substance:	2.934 mg. H_2O, 10.520 mg. CO_2.		
5.243 " "	: 2.475 " AgI.		
$C_{26}H_{34}O_8$.	Calculated.	C 65.79, H 7.22,	OCH_3 6.54.
	Found.	" 65.88, " 7.53.	
	"		OCH_3 6.23.

THE REACTION OF THE MORNING URINE.

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A series of earlier papers from this clinic (1–5) developed the thesis that changes in reaction of the urine voided in the morning depended on the secretion of hydrochloric acid by the stomach, as had been claimed by many other workers (6, 7), and that the presence or absence of a "tide" could be used as an index of the probable presence or absence of free hydrochloric acid in the gastric juice of the subject. With the technique outlined at that time tests have been carried out on over 360 persons. It is the object of the present paper to discuss and illustrate the various types of results found.

The methods used have been described in detail elsewhere (5). Briefly, the plan was to awaken the patient at 7 o'clock, and to collect urine at hourly intervals until 1 o'clock. A meal consisting of two slices of toast with a pat of butter, a glass of milk, a glass of water, and an egg was fed between 8 and 9. The reaction of the urine was determined colorimetrically after 2 cc. of the urine were diluted with 8 cc. of distilled water. Frequently the result of at least one study of gastric acidity, usually obtained by the fractional method (8, 9), was known.

It has already been pointed out that the correspondence between gastric findings and urinary reaction, while generally quite close, is not perfect. One probable explanation of many of the discrepancies lies in irregularities found in determinations of gastric acidity. There are many data available which show that this may vary markedly within a short period of time; in any event successive tests carried out on the same patient indicate that this is true (10, 11). Not infrequently in this research we have met with cases in which a tide was present and a gastric analysis

showed an achlorhydria, but when subsequent gastric analyses were obtained the presence of acid was demonstrated. One case of this nature has been described in detail (12). It is true that in other cases the results definitely did not agree, implying that there is some other factor which must be taken into consideration. The work of McCorvie (13) suggests that the efficiency of the kidneys may be one such modifying factor, but so far the number of proved discrepancies is so small as to furnish no basis for investigation of this phase of the question.

As the work upon the problem of urinary alkalinity progressed it became evident that various distinct kinds of changes in reaction were met with frequently, and that almost all individual studies belonged to one of five types. As the number of results available increased, it seemed evident that there were fairly definite relationships among these types. Forty-eight selected cases are given to show these relationships (Table I).

Group I contains six patients each of whom showed hydrochloric acid in the gastric juice. The changes in urine reaction conform closely to the results expected if the secretion of acid by the stomach is responsible for the alkaline tide, and to figures obtained in the study of various normal subjects carried out by this technique (2, 5). A slight increase in alkalinity occurred during the hour when the meal was fed. There was a slight further change shown in the specimen collected during the following hour, and a marked one in the urine voided 2 hours—2½ hours if the meal was eaten rapidly and was finished more nearly at 8.30 than 9 o'clock—after breakfast. The alkalinity so developed generally persisted during the 5th hour of the test. During the last period, owing probably either to the reabsorption of acid material from the intestinal tract or to the secretion of alkaline juices by the digestive glands, there was an increase in the relative acidity.

Results as exactly regular as those shown in Group I were relatively infrequent in our series. Figures such as those shown in Group II were much more common. The first part of the curve —that up to and including the specimen collected during the hour following the meal—was rather flat; later significant changes occurred which were almost identical with those just discussed. These results in Group II closely resemble the average values

TABLE I.

Selected Examples of Types of Change in Reaction of Morning Urine.

Time of sample.	Group I. Marked gastric tide. HCl present.						Group II. Gastric tide. HCl present.					
	Case No.						Case No.					
	1	2	3	4	5	6	1	2	3	4	5	6
hrs.	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Before meal......	5 2	5 4	5.6	5.5	5.3	5.5	5.5	5.8	5.6	5.6	5.5	5.5
Meal..........	5.5	5 6	6 0	5.8	5.8	6 5	5.5	5.8	5.4	5.8	5.5	5.7
0–1 after..........	6 8	5.8	7 1	6.1	5.9	6.6	5 5	5 6	5.9	5.8	5 4	5.7
1–2 "	7 9	6 5	7.4	7.4	7.2	7 8	7:2	6.5	7 6	6.8	6.2	6 9
2–3 " ...	7.6	5 4	5.6	7.2	7 3	8.1	6.4	7.4	7.4	7.0	6 6	7.6
3–4 "	5.7	5.4	5 5	5.6	5 7	7.6	6 2	6.8	5.9	6.8	5.5	7.4
	Group III. No tide. HCl absent.						Group IV. Respiratory tide. HCl absent.					
Before meal......	5.7	5 3	5.7	5.0	5 4	5 2	5.7	7.6	5 6	6.1	7.3	5.4
Meal..........	5.7	5.2	5 6	5.1	5.3	5 2	5 3	5.1	5.3	5.6	5.3	5.1
0–1 after.......	5.7	5 3	5.6	5.1	5.2	5.4	5 4	4 9	5.3	5.5	5.4	5.2
1–2 "	5.7	5.1	5.5	5.0	5.2	5.3	5.4	4.9	5 4	5 5	5.4	5.2
2–3 "	5 8	5 3	5.7	5.0	5.3	5 3	5 3	5.1	5.2	5.7	5 3	5.2
3–4 "	5 8	5.1	5.5	5.0	5.1	5.4	5.3	5.1	5.4	5.6	5 3	5 1
	Group V. Marked respiratory tide. HCl absent.						Group VI. Two tides. HCl present.					
Before meal.......	8.1	6 8	7.1	7.9	7.2	6.6	7.8	6.2	7.0	7.3	7.6	6.8
Meal..........	8 0	6 2	6 3	7 8	6.9	5.9	6.5	5 9	5.8	5.4	7.4	6.4
0–1 after.........	6 3	6 3	5 6	6 0	6 7	5.8	6.1	5.7	5.2	5.1	5.3	6 5
1–2 "	5 5	5.6	5 7	5.6	6.2	5 5	6 3	6.1	6.8	5 8	5 9	7.1
2–3 "	5 1	5 3	5.9	5 4	5.9	5.3	7.3	7.2	6.5	7 1	7.1	7 2
3–4 "	5 4	5 3	5.8	5.2	5.8	5.2	6 7	6 0	6.5	5.8	7.2	6.4
	Group VII. Probably two tides. HCl present.						Group VIII. Alkaline urine. HCl present.					
Before meal......	8.4	8.0	7.1*	7.6	7.2*	6.8	7.3	7.8	8.0	7.8	7.7	8.2
Meal..............	8 3	8.0	7.1*	7.7	7.2*	7.2	7.5	8.0	8.1	7.2	8 0	7.9
0–1 after..........	6.4	5.6	6.5	6.1	6.1	5.8	7.1	7.5	7.7	8.0	7.5	8 0
1–2 "	7.0	7.1	7.5	7.4	7.2	7.6	6.9	8.2	7.7	7.3	7.6	8.0
2–3 "	7.9	7.4	7.5	7.7	7.0	7.7	7.1	8.4	7 9	7.5	7 8	8.0
3–4 "	7.0	7.2	6.2	7.4	6.8	6.8	6.7	7.2	7.8	8.0	7.6	7.3

* Only one specimen of urine was obtained for the 2 hour period.

obtained upon patients with acid in the gastric juice previously reported from this clinic (5) and similar ones of Ackman (14). The flattening out of the first part of the curve may be due to the modifying influence of respiratory changes to be discussed later or to some unrecognized cause.

Group III contains results representative of many obtained in this series. There was practically no variation in the reaction of the urine during the morning period. Where gastric studies were available in such cases they almost invariably showed an achlorhydria.

Group IV is representative of a rather small number of figures which also were obtained only when an absence of hydrochloric acid was demonstrated in the stomach. It is included because it helps to make clear a larger series of cases discussed in the following paragraph. In Group IV there is shown a relative alkalinity in the specimen collected before the meal was fed, followed by practically constant figures during the remainder of the test. In some instances the patient's urine was obtained during the night preceding the study, and an acid reaction demonstrated in it. The explanation of such findings probably is that suggested by Leathes (15). On awakening there was a "blowing off" of carbon dioxide gas which had accumulated during the night. This period of overventilation resulted in an increased alkalinity of the urine. That such overventilation can cause urinary alkalinity has been demonstrated by Leathes himself and by other investigators (16, 17).

When the alkalinity of the first specimen was marked, the change to an acid reaction appeared, usually, to take place more slowly, giving such figures as are shown in Group V. The result is an apparent acid tide in the morning urine. In our series such changes in reaction were almost invariably associated with an absence of hydrochloric acid in the gastric juice. They did not occur as frequently as did the constant figures illustrated by Group III, but there were enough of them to affect appreciably the form of the average curve in achlorhydria previously published (5). The explanation of them is probably the one already suggested: A relative alkalinity is produced by rapid breathing on awakening, and this alkalinity disappears rather slowly. Two different causes of alkalinity therefore appear to be operative in average

cases during the morning period, as has been previously suggested by the work of Watson (18). One is overventilation and the other secretion of hydrochloric acid by the stomach. It should be possible to show the separate effects of these two factors in the same patient, and the author believes that the cases presented in Group VI are of this kind. There were many results of this general nature in the series, but not many as wholly satisfactory as those given. They show an alkalinity succeeded by a period of quite marked acidity, followed again by a period of alkalinity. In the last specimen there was usually a tendency to return once again to a more acid urine. All portions of each curve contained more than one specimen of urine. The gastric juice of each patient contained hydrochloric acid. The author believes that the first period of alkalinity was produced by respiratory changes, and the second one by the secretion of hydrochloric acid in the stomach. It is evident that the balance between various factors necessary to give such satisfactory results as these must be a very closely adjusted one, and it is not strange that the number of cases showing such figures is small.

Group VII contains a selection of cases which may be closely related to the group just discussed. Here there is only one specimen which shows an acid reaction, while the others are almost uniformly alkaline. There may be no significance at all to such results, for the acidity may be merely an incidental finding which we are not in a position to explain. However, the hour at which the acid specimen was obtained was the same in all cases as that at which the most acid specimen was obtained in the preceding group, and it seems not improbable that the acidity may be attributed to the same cause in both sets of results. Two sets of alkaline curves may have fused, and the single acid specimen may be the only sign of the difference between them. Group VII is introduced here, not because the author wishes to insist upon this explanation of the findings, but because it seems to furnish a logical introduction to the last group of cases presented.

The six cases in Group VIII are representative of a fairly large number in our series. These show an alkalinity which lasts throughout the morning period, although sometimes there was some tendency towards a more acid reaction in the last specimen. Usually the alkalinity was so marked that variations in the figures

could not properly be emphasized, as they might be due to differences in the rate at which carbon dioxide was lost after the specimens were voided (19), rather than to variations in the reaction of the fluid secreted by the kidneys. Hydrochloric acid was demonstrated in the gastric juice of most patients who showed such findings when results of gastric analyses were available. It was present in all the cases illustrated. The explanation which seems to the author to account most satisfactorily for these figures is a complete fusion of the alkaline reactions resulting from the two sources already discussed. Before recovery from the early morning alkalinity—which he believes results from respiratory conditions—had occurred, alkalinity secondary to the secretion of hydrochloric acid by the stomach had commenced. That such curves were not due to food taken the day before the tests, or to infections of the urinary tract, was demonstrated in many instances by studies of the urine of the preceding night. This almost uniformly showed a distinctly more acid reaction than did the specimen obtained just after awakening.

There were of course some tests which did not fit into the series illustrated above in an entirely satisfactory manner. A few of these exceptions should be discussed briefly. It is entirely conceivable that marked sudden variations in urinary alkalinity may occur because of short periods of overventilation. A few cases in which there were changes in reaction apparently due to this cause were found, but the number was very small. In a series of studies of sanitarium patients who could not be kept under strict control we expected more irregularities of this sort than we encountered.

In another small group a relative alkalinity developed during the 2nd hour of the test—the one when the meal was fed—and was not followed by any further increase in the degree of alkalinity. It seems probable that such results might arise from a delay in the respiratory tide, for the subjects might have slept through the 1st hour of the morning and resumed their daily activity only during the 2nd one. As a matter of fact in all of the cases in which such results were found there was hydrochloric acid in the gastric juice, and therefore the figures may represent some sort of an unusual response to acid secretion by the stomach.

There was one small group in which an alkalinity developed in the course of the morning which generally seemed to be associated with an absence of hydrochloric acid in material withdrawn from the stomach. In these cases the increase in alkalinity occurred at the very end of the period. It is possible that in them the alkaline material taken with the breakfast was excreted and caused the change in reaction, but the number of patients who showed this finding was so small, and the number of those suffering from achlorhydria who showed nothing of the kind was so large, that the explanation cannot be regarded as satisfactory. Another possibility is that this group contains those who stayed in bed until dinner time and then got up. It seems to the author that such a procedure might give rise to the findings, for the rate of pulmonary ventilation probably increases under such conditions.

SUMMARY.

This study indicates that there are two factors which must be taken into consideration to explain changes in reaction of the morning urine. One is the secretion of hydrochloric acid by the stomach which causes the development of an alkalinity after a meal is fed. The other is, probably, an adjustment of the respiration to waking conditions which frequently shows its effect very early in the morning. If the interplay of these two factors is kept in mind, most results can be understood.

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VARIATIONS IN THE MORNING ALKALINE TIDE OF NORMAL INDIVIDUALS.

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In previous communications (1–5) evidence was presented tending to show that variations in the kind of breakfast eaten influenced the reaction of specimens of urine collected during the morning, and that these variations appeared to correspond with the reported effect of such meals upon the degree of gastric acidity. Papers having a similar import have appeared from other clinics since these articles were written (6, 7), but some observers have met with findings which are quite different (8–10). A paper of particular interest is that of Muschat (11) who in experiments upon a normal man studied the urine throughout the day and found that the "tides" tended to persist unchanged regardless of what meals were eaten or the times at which they were taken. Since these discrepancies have been reported, and since in the earlier work from this institution results seemed to be distinctly regular in their nature, it was deemed best to carry out experiments similar to those previously presented on more normal subjects. In the present paper there are reported some studies which show that comparable experiments upon different normal people may produce results which are quite unlike in nature.

Under the heading Subject I (Table I) all the experiments carried out on E. G. A., a normal man who was doing light laboratory work during the investigations, are recorded. Many, but not all, of the figures have been reported previously. The reactions of urine specimens collected for an hour before any meal was taken, for an hour during which the different meals were fed, and for hourly periods during the remainder of the morning are shown. The tests are arranged in the order in which they were

TABLE I.

Repeated Tests of Changes in Urinary Acidity of Normal Subjects

Time of sample	Subject I, E.G A, normal man. Test No 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
hrs	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Before meal	5 3	6 6	5 7	5 2	5 4	5 2	6 5	5 7	5 4	5 5	5 4	5 6	5 7	5 6	5 6
Meal	5 3	6 9	5 8	5 3	5 3	5 4	5 7	5 8	6 2	5 6	5 4	5 6	5 7	5 7	5 6
0–1 after	5 3	6 9	6 3	5 3	5 5	6 8	6 3	6 4	7 5	5 8	5 3	6 6	6 6	6 9	5 8
1–2 "	5 2	7 2	6 7	5 2	5 7	7 2	6 9	6 5	7 8	6 5	5 0	6 7	7 0	6 6	5 7
2–3 "	5 1	6 5	6 6	5 1	5 6	6 3	6 6	6 0	7 4	5 6	5 2	5 7	6 5	5 7	5 8
3–4 "		6 0	5 7	5 1	5 3	5 4	5 8	5 6	6 8				5 4		

	Subject II, R S H, man. Test No 1	2	3	4	5	6	7	8	Normal women. Subject III Test 1 W	Subject III Test 2 S	Subject IV. Test 1 W	Subject V Test 1. W
Before meal	5 7	5 6	5 4	5 6	5 5	5 4	5 6	5 5	5 5	5 5	8 0	7 8
Meal.	6 7	5 8	5 4	5 6	6 2	5 8	5 7	5 9	5 4	6 8	8 4	7 5
0–1 after.	7 4	7 3	5 6	6 5	7 4	5 9	5 5	6 9	5 7	6 4	8 0	7 2
1–2 "	7 1	7 4	6 0	6 4	7 4	5 6	5 3	6 9	5 6	7 5	8 0	7 7
2–3 "	6 8	6 2	5 6	6 6	7 0	5 6	5 3	6 7	5 4	7 3	8 4	7 4
3–4 "		5 6	5 4	6 2	6 3	5 6	5 3	6 4	5 4	7 1	8 2	

	Subject VI, T M S, normal man. Test No 1	2	3	4	5	6	7	8	9	10
	W, up	R, up	O, abed	W, abed	S, up.	S, abed	S, up	O, up	R, up	R, abed
Before meal.	5 4	5 3	5 5	5 2	5 2	5 3	6 0	5 8	6 1	5 2
Meal	5 8	5 3	6 5	6 1	5 2	6 1	6 9	6 9	6 2	5 8
0–1 after.	6 0	5 6*	7 3	6 4	5 4	7 0	7 4*	6 5	6 6	6 1
1–2 "	5 4	5 6*	7 4	7 0	5 4	7 5	7 4*	6 7	6 0	7 2
2 3 "	5 3	7 3	7 5	7 3	5 7	7 7	7 7	6 9	6 9	
3–4 "	5 4	6 7	7 3	6 4	6 0	7 5	7 5	7 1	7 1	

O denotes no food or water taken; W, two and a half glasses of water only; R, regular breakfast described in the text; S, regular breakfast plus beefsteak

* Only one specimen was collected during the 2 hour period.

carried out. It is evident that, with the exception of four tests, there was regularly a decrease in acidity after the meal was eaten. The degree of this change varied somewhat in each experiment, and in an earlier paper (2) attention was called to the fact that these variations corresponded with differences in the carbohydrate and protein content of the different breakfasts eaten. An examination of the régime on the days when changes in alkalinity (tides) did not occur seems to give an entirely adequate explanation for most, if not for all, of these four experiments. The tide was absent in Tests 1, 4, 11, and 15. In Test 1 no food was taken and no water drunk; on the day when Test 4 was carried out two and a half glasses of water alone were taken at the usual breakfast time; in Test 15 nourishment consisted only of the same amount of water plus two slices of bread. Test 11 is the experiment described in detail by Hubbard and Allen (4) and must be discussed at somewhat greater length. A stomach tube was passed before a breakfast low in protein and high in carbohydrate—consisting of dates, Shredded Wheat, and sugar—was eaten. Of eight gastric specimens taken through the tube during the experiment only one contained free hydrochloric acid, and when titrated to the end-point with Töpfer's reagent this showed a concentration of only 18 cc. of 0.1 N acid per 100 cc. of gastric juice. Distinctly high values for the titration to phenolphthalein (the highest value for total acid found was 110 cc. of 0.1 N) make the interpretation of the results uncertain. Since in the other experiments except these four adequate breakfasts were eaten and satisfactory tides obtained, the series seems to show clearly that in this subject variations in the alkaline tide could be properly attributed to variations in the amount of acid secreted in the stomach.

Similar conclusions may also undoubtedly be drawn from a study of the results upon Subject II. Part, but only part, of these figures has been published elsewhere. Six of the eight tests show tides, and the two which do not are adequately explained by the experimental conditions. The figures given in Test 6 were obtained when no meal was eaten and no water drunk, while in Test 8 the breakfast eaten consisted entirely of sweet chocolate. On other occasions meals of a more normal type were taken.

The results upon Subject III, a nurse who was pursuing her duties during the tests, can also be explained on the basis of varia-

tions in gastric secretion. There was no significant tide in Test 1 when water only was taken for breakfast, but in Test 2, when a substantial meal was eaten, a well marked tide was present.

Subjects IV and V showed something very different. Both were normal women, and both received water only for breakfast. A rather marked degree of alkalinity was present in the first specimen obtained, and this persisted through the morning. Such results have been mentioned by Watson (8) and discussed in a previous paper dealing with the interpretation of the alkaline tide (5), but their occurrence in normal subjects has not been strongly emphasized. It is difficult to give an entirely satisfactory explanation for them. The night urine preceding each of the tests was collected, and an acid reaction found in each; the readings were 5.6 and 5.8 pH for Subjects IV and V respectively. It seems improbable, therefore, that the alkalinity on the mornings of the experiments was due to the diet eaten earlier. The results may be due, as has been suggested previously for a slightly different group of cases (5), to an exaggeration of the effect described by Leathes (12). There may have been a "blowing off" of the acid carbon dioxide gas on awakening which produced an alkalinity similar to that observed in any form of overventilation. In such subjects as the two under consideration this may persist throughout the morning for some unknown reason. The author believes it possible that the cause is a secretion of acid by the stomach, which takes place even when no food is taken. In any event it seems improbable that further urinary alkalinity could be superimposed upon such a base-line as the result of acid secretion by the stomach, for there seems to be some sort of a limiting alkalinity below which the reaction of the urine does not readily drop. This limit is due, at least in part, to the chemical nature of the fluid (13).

None of the cases described up to this point offers a contradiction to the thesis supported in the earlier papers. None of them resembles the experiments described by Muschat and others in which the alkaline tide—defined as an increased alkalinity which developed during the morning period—persisted notwithstanding marked variations in the diet. Subject VI however showed such results clearly. This subject, T. M. S., was a recent medical graduate who was serving as an interne. He took at different times and under different conditions the meals which have been

most studied in this series of experiments. These consist of the regular breakfast (designated R in Table I) consisting of two slices of toast, a glass of milk, a glass of water, a pat of butter, and an egg; the same breakfast plus beefsteak (S); two and a half glasses of water (W); and no food and no drink (O). In some of the experiments he remained in bed, and in others he got up as usual and pursued his regular duties. The activity involved in these was approximately equivalent to the laboratory work of E. G. A. (Subject I).

A study of the results shows that in this instance the changes in reaction known as the tide persisted under all conditions, just as they have in part of the work of other observers. There were no essential differences which could properly be attributed to variations in the amount of acid secreted by the stomach or to the occupation of the subject. When he was in bed, and irregularities in ventilation should be reduced as nearly to a minimum as can be accomplished experimentally, the results were in general the same as when he was carrying out his routine work. There was some variation among the results of the different tests, but this certainly cannot be attributed either to activity or to the meals used. In Test 1, in which water only was taken, the change in reaction was less marked than in most of the others, but this observation is counterbalanced by Test 8, in which neither food nor fluid was used, and in which the change in reaction was practically as marked as it was when ample breakfasts were eaten. Test 5 is the one which varies most widely from the other results and from the figures usually obtained in our studies. It is decidedly unlike Test 7, for example, which was supposed to be carried out under exactly similar conditions. The only difference which could be made out between these two experiments was that on the night before the earlier, more unusual, one the subject did not go to bed until 4 o'clock in the morning, while before the later one his retiring time was at a more usual hour. Whether this explains the absence of a significant tide after a meal which stimulates gastric secretion as much as does a breakfast containing both egg and beefsteak, remains an open question.

It is not possible to decide whether in this case there was a secretion of hydrochloric acid by the gastric glands which persisted regardless of the food stimulus furnished, or whether there was a

fairly definite rhythm of changes in the reaction of the body fluids which was maintained in spite of variations in the stomach secretion. In view of the association between gastric acidity and the alkaline tide illustrated in the earlier papers and in the first part of this one, the authors incline to the former of these two hypotheses. They believe, too, that such evidence as can be adduced in favor of gradual adaptation of urinary reaction in prolonged dietary experiments (7) tends to support this view.

SUMMARY.

In this paper the effect of variations in foods upon the reaction of urine secreted by normal subjects after a meal has been discussed. All subjects do not respond alike in such experiments. In some the changes in reaction correspond closely to those which would be expected if gastric secretion induced by foods caused a relative alkalosis in the body and a consequent alkalinity of the urine, for when food is omitted the changes do not occur. In others an increased alkalinity of the urine develops whether food is taken or omitted. In these there seems to be an acid-alkali rhythm which does not depend on the immediate stimulation of the gastric glands, but which may be secondary to a secretory rhythm of the stomach. In still others there is a marked alkalinity which develops before any meal is eaten and which may persist throughout the entire morning period, even when food is omitted.

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GASOMETRIC DETERMINATION OF METHEMOGLOBIN.

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In the present paper the method of Van Slyke (1) for methemoglobin determination has been modified by employing technique developed by the writers (2) for determining the carbon monoxide-binding capacity of blood. The principle, as in the former methemoglobin method (1), is that introduced by Nicloux and Fontes (3). Two determinations are required. In one (*A*) the normal or active form of hemoglobin, capable of binding O_2 and CO, is determined by measuring the CO-binding capacity of the hemoglobin-methemoglobin mixture. In the other (*B*) sodium hydrosulfite is added, changing methemoglobin into active reduced hemoglobin, and the total hemoglobin is determined by the CO-binding capacity. The difference, $B - A$, indicates the methemoglobin. The technique here introduced has the advantage that all the operations, reduction with hydrosulfite, saturation with CO, and determination of CO bound by hemoglobin, are carried out in the chamber of the Van Slyke-Neill apparatus (4, 5). In consequence, the procedure is more simple and rapid than that previously presented (1), and requires much less blood, as little as 0.2 cc., or even 0.1 cc., sufficing for an analysis.

Conant, Scott, and Douglass (6) have shown that the hydrosulfite-CO procedure is not applicable in the presence of hematin, because the latter is reduced and behaves like methemoglobin in binding CO. They use titanous tartrate to reduce the methemoglobin, and determine the O_2-, instead of the CO-, binding capacity. Their procedure is not affected by the presence of hematin. The occurrence of hematin in blood is rare, however, except under experimental conditions designed to produce it. We have accordingly for simplicity retained the hydrosulfite-carbon monoxide procedure.

In determining the carbon monoxide capacity in blood reduced by hydrosulfite, the only modification necessary to our former procedure (2) for hemoglobin determination is the use of higher CO tensions to saturate the hemoglobin. The presence of hydrosulfite and ammonia appears to lower somewhat the affinity of reduced hemoglobin for CO, so that 100 mm. tension of the latter, instead of only 30, are required to insure complete conversion of the hemoglobin to carboxyhemoglobin.

Reagents.

Nicloux's ammoniacal sodium hydrosulfite solution is prepared as previously described (1). The *carbon monoxide gas, 1 N air-free sodium hydroxide,* and *5 N sodium hydroxide* are prepared and handled as outlined by Van Slyke and Hiller (2).

The *acid ferricyanide solution* is prepared as follows, with more acid than formerly. To 92 volumes of a stock solution containing 32 gm. of $K_3Fe(CN)_6$ per 100 cc. are added 20 volumes of concentrated lactic acid, of specific gravity 1.2. The ferricyanide in this acidified solution undergoes slow decomposition, but if kept out of direct sunlight can be used for about 2 months.

Procedure.

Determination of Active Hemoglobin.—The carbon monoxide capacity method of Van Slyke and Hiller (2) is used without change.

Determination of Total Hemoglobin. For 2 Cc. of Blood.—2 drops of caprylic alcohol are drawn into the capillary beneath the cup of the manometric apparatus. Into the cup are measured 4.3 cc. of water. With a stop-cock pipette provided with a rubber tip (see Fig. 4, p. 532, of Van Slyke and Neill (4)), 2 cc. of blood are run directly into the chamber, followed by a few drops of the water in the cup to wash the blood through the capillary. From a micro burette[1] 0.4 cc. of the ammoniacal sodium hydrosulfite solution is run into the chamber, followed by the remaining water in the cup. 1 or 2 cc. of mercury are placed in the cup above the

[1] The micro burette used for measuring the hydrosulfite and the acid ferricyanide was made by sealing a stop-cock onto a pipette graduated in 0.01 cc. divisions. The delivery capillary was provided with a rubber tip as shown in Fig. 3, p. 125 of Van Slyke's paper (5) and described on p. 126.

chamber. Carbon monoxide sufficient to give 150 mm. of pressure is measured into the chamber from a modified Hempel pipette in the manner described on pp. 816 and 817 of our previous paper (2).

The *equilibration of the blood solution* with *CO* is carried out as described on p. 811 of our former paper (2), except that a little more time seems necessary. We shake the chamber 1.5 instead of 1.0 minute.

The *determination of CO bound as HbCO* is also carried out in all details, including the *c* correction, as described on the same page, except that 0.3 cc. instead of 0.25 cc. of acid ferricyanide solution is added. The value of *c* in the present procedure is somewhat greater than in the carbon monoxide capacity method (2), because of the greater amount of CO physically dissolved by the blood solution at the higher CO pressure used for saturation. The value of *c* which we find in our laboratory, with a temperature of 20–25° is about 14.0 mm. Each analyst should, however, determine it for himself repeatedly.

The cleaning of the chamber after each analysis is more important in this analysis than in the simple carbon monoxide capacity determination, because in the present case any particle of methemoglobin ferricyanide precipitate left adhering to the walls of the chamber will be reduced in the next determination to active hemoglobin by hydrosulfite, and added to the total hemoglobin found. A little of the hydrosulfite solution added to the first portion of water used to clean the apparatus assists in dissolving such particles quickly. The procedure for rapid and complete washing of the apparatus is described on p. 813 of our former paper (2).

For 1 Cc. Blood Samples.—The procedure is the same as that used for 2 cc. samples except that half as great a volume of each reagent is used for 1 cc. of blood as for 2 cc. The pressure of CO used is the same, 150 mm.

Micro Determination of Total Hemoglobin with 0.1 Cc. or 0.2 Cc. Blood Samples.—The procedure for measuring the blood and transferring it to the chamber of the apparatus is the same as that described on p. 814 of our previous paper (2), except that 0.05 cc. of the ammoniacal sodium hydrosulfite solution is run into the chamber of the apparatus before the final washing of the cup so

that this volume of fluid is part of the total 2 cc. measured into the chamber. Carbon monoxide to 150 mm. pressure is admitted into the evacuated chamber in the same manner described above. Of the acid ferricyanide solution only 0.05 cc. is added. It is run in while the blood solution is still in the top of the chamber and is followed by several drops of mercury which break up the methemoglobin precipitate into fine particles. The procedure is continued as detailed on p. 815 of our former paper (2).

The c correction for the micro method is somewhat over 20 mm. It must be redetermined with each set of micro analyses.

The *calculations* for total hemoglobin are the same as those described in the former paper (2) on pp. 812 and 813.

$$\text{CO capacity} = (p_1 - p_2 - c) \times f.$$

For 2 cc. blood samples f is a factor from the last column of Table II or III of Van Slyke and Neill (4). For 1 cc. samples f is found in the seventh column of their Table II or III, when S is 3.5 cc. and a is 2.0 cc., or in the sixth column when the final gas pressure is read with the gas at 0.5 cc. volume. When 0.2 cc. of blood is employed f is found in the fifth column of their Table II or III. When the sample is 0.1 cc. the factors used for 0.2 cc. are multiplied by 2.

$$\text{Methemoglobin} = \text{total hemoglobin} - \text{active hemoglobin}.$$

EXPERIMENTAL.

Tension of CO Required for Complete Conversion of Reduced Hb into HbCO.—The material used was blood in which part of the hemoglobin had been oxidized to methemoglobin by addition of measured amounts of ferricyanide. The amount of ferricyanide taken was 0.5 mol per mol of hemoglobin present (1 mol of Hb being estimated as the amount binding 1 mol of O_2 or CO). As would be predicted from the results of Conant and Fieser (7) this procedure produces a mixture of approximately equal parts of active hemoglobin and methemoglobin.

To 50 cc. of blood with 18.41 volumes per cent of CO-binding capacity Merck's saponin was added with shaking until hemolysis was complete, then 0.3 cc. of a solution containing 32 gm. of potassium ferricyanide per 100 cc. was added. 2 cc. portions of

TABLE I.

Tension of CO Required for Quantitative Saturation of Blood for Total Hemoglobin Estimation.

CO capacity of blood before oxidation.	Tension of CO at beginning of saturation.	CO bound per 100 cc. of treated blood.	
vol. per cent	*mm.*	*cc.*	*per cent CO bound by untreated blood*
18.41	35	17.25	93.7
	70	18.23	99.0
	100	18.50	100.5
	150	18.44	100 2

TABLE II.

Macro and Micro Determinations of the Methemoglobin Content of Blood.

Quantity of blood used for analysis.	CO capacity of blood before oxidation	Total hemoglobin in terms of CO capacity.	Unoxidized hemoglobin in terms of CO capacity.	Methemoglobin in terms of CO capacity.
cc.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
2	18.41	18.34	9.01	
	18 40	18.51	8.93	
		18.47		
Average.....	18.41	18.44	8.97	9.47
1		18.32	8.82	
		18.38	8.96	
		18.46		
Average.....		18.39	8.89	9.50
0 2		18.40	8.76	
		18.15	8.83	
		18.51		
Average.....		18.35	8.80	9.55
0 1		18.67	8.53	
		17.81	8.76	
		18.35		
Average.....		18.28	8.65	9.63

this blood were analyzed for total hemoglobin as described above, by treatment with hydrosulfite and CO. The procedure was varied, however, with respect to the CO tensions used to saturate

the blood in the chamber. The results, given in Table I, show that 100 mm. of CO tension are adequate.

Constancy of Results.—The same ox blood was partially oxidized with ferricyanide in the same manner. The mixture was immediately analyzed for total hemoglobin and for active hemoglobin. Determinations were carried out with 2 cc., 1 cc., 0.2 cc., and 0.1 cc. samples. The results recorded in Table II indicate the order of constancy obtained by the method.

SUMMARY.

The gasometric method for the determination of methemoglobin has been simplified by adapting to it the carbon monoxide capacity technique of the authors. The entire procedure is carried out in the manometric apparatus of Van Slyke and Neill. Amounts of blood varying from 2 cc. to 0.1 cc. can be used for the analyses.

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GASOMETRIC CONTROL OF STANDARD SOLUTIONS FOR THE PALMER HEMOGLOBIN METHOD.

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The carbon monoxide capacity determination previously published by the writers (1) is utilized to determine the hemoglobin content of the 5-fold diluted blood solution used as stock standard in the colorimetric Palmer hemoglobin method. It is necessary to saturate this blood solution with carbon monoxide instead of illuminating gas, because some constituents of the latter diminish the accuracy of the analysis.

The only criticism to which, in our experience, the Palmer method has been open, has been the tendency of the standards to deteriorate. We believe that the method here presented affords an accurate and direct means for detecting deterioration, and that the observations recorded in the experimental part of the paper indicate the conditions for keeping deterioration at a minimum.

Method.

Hemoglobin Solutions.—The stock hemoglobin standard is made up by approximately 5-fold dilution of analyzed blood as described by Palmer (2) except that instead of saturating with illuminating gas about 2 liters of pure CO[1] are bubbled slowly through the solution. The standards are kept in dark bottles preferably in an ice box, with cork or glass stoppers sealed with paraffin. Each time the bottle is opened CO gas is again bubbled through the solution for a short time before the stopper is sealed.

When the dilute 1 per cent standards for colorimetric estimations

[1] The preparation of pure CO is described in our paper on carbon monoxide capacity method (1) p. 808 and Fig. 2, p. 810.

are prepared from the stock standards, illuminating gas may be used in order to saturate with CO. The other constituents of the gas do not affect color.

Reagents.—The reagents are the same as those described for the macro carbon monoxide capacity method (1) with the exception of the acid ferricyanide solution. This is made by mixing 92 volumes of a solution containing 32 gm. of $K_3Fe(CN)_6$ per 100 cc. with 52 volumes of concentrated lactic acid of specific gravity 1.2. Although slow decomposition takes place, such a solution not exposed to direct sunlight can be used for 2 months.

Analysis.—2 drops of caprylic alcohol are drawn into the capillary beneath the cup of the manometric apparatus (3, 4). In the cup is placed about 1 cc. of mercury. With a stop-cock pipette provided with a rubber tip (see Fig. 4, p. 532, of Van Slyke and Neill (3)), 10 cc. of the Palmer hemoglobin standard are run into the chamber under the mercury. Through the same mercury seal 2 cc. of CO are added as described in the carbon monoxide capacity method ((1), p. 810). The procedure continues as described from pp. 810–813 (1), except that 0.36 cc. of the acid ferricyanide described above is used to liberate the CO bound as HbCO, and 1.5 cc. of air-free 1 N NaOH are used to absorb the CO_2. The absorption of CO_2 takes somewhat longer, hence it is important to note the fall of mercury in the manometer. When the mercury ceases falling, agitate the chamber slightly with the hand. If no further change occurs in the manometer meniscus the absorption is completed.

The *c* correction is established by carrying out the procedure in the same manner, except that 10 cc. of an aqueous solution containing 4 cc. of strong ammonia in 1 liter are substituted for the hemoglobin standard. This correction was found by us to be constant at 7.8 mm. between 20–25°. Each analyst should establish his own *c* correction repeatedly.

The calculation is the same as that described in the carbon monoxide capacity method (1). The factors used for the Palmer hemoglobin standards are given in Table I.

EXPERIMENTAL.

Five Palmer hemoglobin standards were made up from various samples of analyzed ox blood. Each sample was diluted to have

a carbon monoxide capacity of $\frac{18.5}{5}$ volumes per cent. Portions of Standards II, III, and IV were set aside without either CO or illuminating gas being passed through the solutions. All other standards were saturated with pure CO. Their carbon monoxide capacities were determined when the solutions were prepared and

TABLE I.

Factors by Which P_{CO} is Multiplied to Calculate Strength of Palmer Standard Hemoglobin Solutions.

Temperature.	Factors to calculate vol. per cent CO capacity of hemoglobin solution. $a = 2.0$
°*C.*	
15	0.02502
16	492
17	484
18	474
19	464
20	0.02456
21	448
22	438
23	430
24	420
25	0.02412
26	404
27	396
28	386
29	378
30	0.02370
31	362
32	354
33	346
34	338

at intervals thereafter, by the method described above. The standards were stored in dark bottles with paraffin-sealed stoppers and kept in the ice box at about 7°. The results of these analyses are shown in Fig. 1.

Standard I had not changed after a period of 32 weeks. Standards II and IV were unchanged after 17½ weeks. The portions of

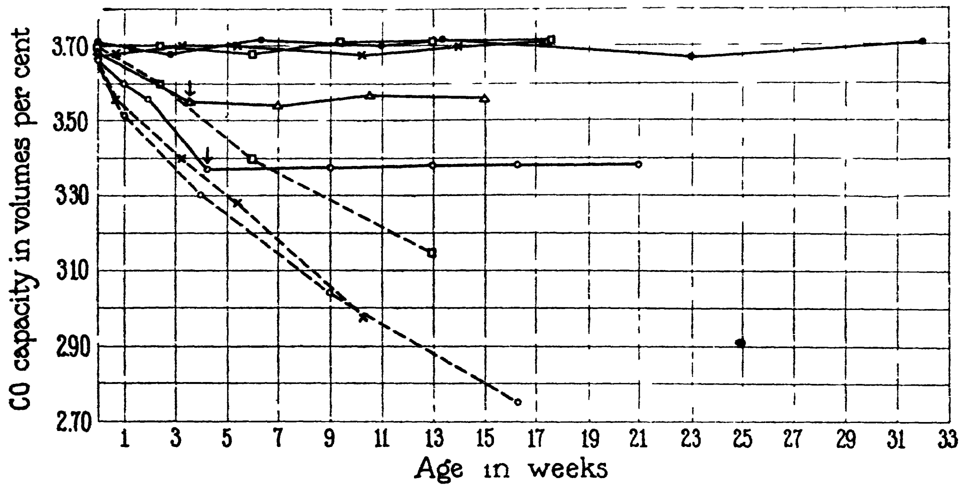

FIG. 1. Change in Palmer standard hemoglobin stock solutions. All were kept at 7° temperature, with other conditions varying as indicated. ●——● Hemoglobin Standard I saturated with CO. ×——× Hemoglobin Standard II saturated with CO ○——○ Hemoglobin Standard III saturated with CO. □——□ Hemoglobin Standard IV saturated with CO. △——△ Hemoglobin Standard V saturated with CO. ×-----× Hemoglobin Standard II no CO. ○-----○ Hemoglobin Standard III no CO. □-----□ Hemoglobin Standard IV no CO. The arrow indicates the point at which Standards III and V were re-saturated with CO after each opening of bottle for removing samples.

Standards II, III, and IV which were not saturated with CO showed a steady decrease in CO capacity, as shown by the dotted line curves in Fig. 1. Standard II fell to 2.98 volumes per cent in 10 weeks, Standard IV to 3.15 volumes per cent in 13 weeks, and Standard III to 2.75 volumes per cent at the end of 16 weeks. Standards III and V, which had been saturated with CO at the time they were prepared, were opened at frequent intervals without resaturation. These showed a decrease in CO capacity almost as great as the solutions which had had no CO. After 4 weeks these solutions were resaturated with CO, and thereafter they were resaturated each time the bottles were opened for removing samples. From this point the CO capacities remained constant. Standard III was examined thus for 21 weeks and Standard V for

TABLE II.

Gasometric and Colorimetric Hemoglobin Content of Palmer Hemoglobin Standards.

Standard No.	Age.	By gasometric analysis.	By colorimetric comparison with unaltered standard solution.
	wks.	*vol. per cent*	*vol. per cent*
II	17	3.71	3.70
IV	17	3.71	3.70
III	21	3.38	3.54
V	15	3.56	3.60

15 weeks. These experiments show the importance of keeping the solutions saturated with CO.

At the end of the periods of observation 1 per cent standards were prepared as described above and compared in the colorimeter with Standard I as the basis of comparison. Those standards which had kept a constant CO capacity also proved colorimetrically exact. Those which had not been saturated with CO at any time gave 1 per cent standards which did not match the CO standards in color. There was such a predominance of yellow that the color intensities could not be matched with accuracy. Standards III and V, which had decreased in CO capacity during the early period of observation and had then become constant at a lower value, on saturation with CO also showed lower values colorimetrically. These results are shown in Table II.

It is obviously important to keep the Palmer hemoglobin standards saturated with CO so that they may keep both their original CO capacity and their color. In order to insure accurate results in the colorimetric estimations, the CO capacity of the standards should approximate closely 3.70 volumes per cent and the 1 per cent standard should be renewed every 2 weeks, as suggested by Palmer (2). If it is kept longer, it often fades or develops a yellow tinge which makes it difficult to match with a freshly prepared hemoglobin solution.

SUMMARY.

A method is described for estimating the carbon monoxide capacity of the hemoglobin standards used in the colorimetric estimation of hemoglobin described by Palmer.

When these standards are kept saturated with carbon monoxide, and in the dark at 7°, they have been found to remain quantitatively unchanged in either carbon monoxide capacity or color value for periods up to 32 weeks.

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GASOMETRIC DETERMINATION OF OXALIC ACID AND CALCIUM, AND ITS APPLICATION TO SERUM ANALYSIS.*

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The reaction between oxalic acid and permanganate indicated by the equation

$$5H_2C_2O_4 + 2KMnO_4 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 8H_2O + 10CO_2$$

is the basis of most of the calcium methods current in biological chemistry, the amount of oxalate in precipitated calcium oxalate being determined by titrimetric measurement of the permanganate utilized. In this paper we present results showing that the oxalate can be determined with equal accuracy by measurement in the Van Slyke-Neill (1924) manometric apparatus, and that the gasometric procedure can be applied with especial advantage to micro determination of serum calcium.

The advantage of the gasometric method in micro analyses is indicated by the fact that the oxalate precipitated by 0.1 mg. of Ca, the amount in 1 cc. of ordinary serum, reduces in titration only 0.50 cc. of the 0.01 N permanganate used in the Kramer and Tisdall (1921) method, while by the gasometric procedure the CO_2 produced exerts at 0.5 cc. a pressure of about 160 mm. to read on the Van Slyke-Neill manometer. The permanganate without unusual precautions can hardly be measured with better than 4 per cent accuracy, while 1 per cent is easily attainable in the manometric measurement. With larger amounts of oxalate to determine, titration and gasometric measurement yield precise and identical results.

* A preliminary note on the method appeared 3 years ago (Van Slyke and Sendroy, 1926)

Because of the difficulty of obtaining precise results with the permanganate titration in micro analyses Hamilton (1925) and Trevan and Bainbridge (1926) have introduced an extra step. They heat the calcium oxalate precipitate until it is changed to calcium oxide (Hamilton) or carbonate (Trevan and Bainbridge), dissolve the residue in excess hydrochloric or phosphoric acid, and titrate back with alkali. The procedure is less simple than the direct permanganate titration, and presumably for that reason the latter appears to continue in general use.

The demonstration of a quantitative yield of CO_2 from oxalic acid treated with excess permanganate in the manometric apparatus offered no difficulties. The technique for obtaining, pure and without loss, the minute amounts of calcium oxalate crystals which the delicacy of the manometric method permits one to determine has absorbed the greater part of the time required for the work here presented.

The preliminary treatment of blood serum or plasma proved to be a point of importance. Halverson and Bergeim (1917), of whose blood calcium method most subsequent ones are more or less successful modifications, precipitated the proteins with picric and hydrochloric acids, and brought the filtrate to the proper slightly acid reaction by adding sodium acetate and ammonia. Rothwell (1927) has followed a similar procedure. Other authors, however, (Clark, 1921; Kramer and Tisdall, 1921; Clark and Collip, 1925; Hamilton, 1925; Trevan and Bainbridge, 1926) have precipitated the calcium in the whole serum slightly diluted. In our preliminary note (1926) we also followed this procedure.

Results reported below, however, (see Table IV) indicate that the serum proteins inhibit measurably the precipitation of calcium oxalate. By precipitation in diluted serum we have obtained results 5 to 15 per cent lower than those obtained by precipitation in the protein-free trichloroacetic acid filtrate. It was further noted that after the calcium had been precipitated as completely as possible by ammonium oxalate in diluted serum, when the supernatant mother liquor was deproteinized with trichloroacetic acid and the filtrate brought to pH 5 an additional precipitate of calcium oxalate appeared. Quantitatively it made up the 5 to 15 per cent deficit noted above. Accordingly, in our method we pre-

cipitate the calcium in the trichloroacetic acid filtrate rather than in diluted serum.

Another point of technique, and the one that has drawn most attention from previous authors, has been the washing of the calcium oxalate precipitate. As pointed out by Clark (1921) the solubility of calcium oxalate, though slight (7 mg. per liter at 25°), is sufficient to cause significant losses when only 0.2 to 0.4 mg. of precipitate is washed. 10 cc. of water, if in contact long enough to become saturated (which it is not, of course, in any ordinary washing technique), could dissolve 0.07 mg. of the precipitate. When the washing is done by the centrifugation method, there is also opportunity for loss of some crystals in the decanted washings. The losses that can readily occur are exemplified in Table III. Obviously a minimum of washing is desirable. Yet it must be sufficient to remove the ammonium oxalate completely, or so nearly so that the trace left will just compensate for the oxalate lost. The washing procedure empirically found to meet this requirement, will, as stated by Clark and Collip (1925) be "arbitrary and must be closely adhered to." Kramer and Tisdall wash by centrifugation three times with 4 cc. portions of dilute ammonia, syphoning off the washings; Clark and Collip wash only once with 3 cc., but obtain more complete removal of mother liquor and washings by permitting the inverted tube to drain for 5 minutes. After numerous trials of similar procedures we finally adopted two washings with 3 cc. portions of dilute ammonia, poured upon the precipitate but not stirred up with it. This technique gave in our hands the most consistent results (*e.g.* see Table V). However, we consider that the washing process remains the part of the analysis to which its maximum error (±3 per cent) is chiefly due. Any errors beyond the above limit readily occur if the washing procedure is not carried out scrupulously as directed.

Reagents.

Trichloroacetic Acid, 20 Per Cent.—20 gm. dissolved and diluted to 100 cc., freshly prepared for use.

Sodium Acetate, 20 Per Cent.—20 gm. dissolved and diluted to 100 cc.

Brom-Cresol Green, 0.016 Per Cent.—Prepared from stock solutions as described by Sendroy and Hastings (1929).

1:1 Ammonia Water.—Concentrated ammonium hydroxide diluted to twice its volume.

Ammonium Oxalate.—Saturated aqueous solution, about 3.5 per cent.

Ammonium Hydroxide, 2 Per Cent.—2 cc. of concentrated ammonium hydroxide diluted to 100 cc.

Approximately 1 N Sulfuric Acid.—27 cc. of concentrated H_2SO_4 diluted to 1 liter.

Approximately 0.15 N Potassium Permanganate.—4.8 gm. of $KMnO_4$ are dissolved and diluted to 1 liter. A portion is acidified before use by the addition of 0.05 volume of 1 N H_2SO_4.

Approximately 5 N Sodium Hydroxide.—200 gm. of NaOH dissolved and diluted to 1 liter. The solution is conveniently used from a tube with a pinch-cock at the bottom, described by Van Slyke and Neill (1924, p. 535) without oil.

All reagents, water, and filter paper should be tested for calcium as an impurity.

Procedure.

Deproteinization.—When there is sufficient material 2 or more cc. of serum or plasma are placed in a measuring flask calibrated to hold 5-fold the volume of the sample. The latter is diluted with about 3 volumes of water, and then 1 volume of a freshly prepared 20 per cent trichloroacetic acid solution is added drop by drop. Water is added up to the mark; the material is mixed and allowed to stand ½ hour for precipitation to finish. The mixture is then transferred to a tube and centrifuged. The supernatant liquid is poured off through a small ashless filter paper, and a filtrate is obtained of about four-fifths of the mixture volume.

When only 1 cc. of serum is available, it is precipitated in a 10 cc. flask with 2 cc. of 20 per cent trichloroacetic acid, and as much of the filtrate as possible used for analysis.

Precipitation of Calcium Oxalate in the Filtrate.—To a measured portion of 5 or 10 cc. of filtrate in a scrupulously clean[1] 15 cc. graduated centrifuge tube 1 cc. of 20 per cent sodium acetate,

[1] When the tubes are not in use we keep them immersed in cleaning fluid (1 gm. potassium dichromate per 100 cc. of concentrated sulfuric acid).

6 to 8 drops of 0.016 per cent brom-cresol green indicator, and 1 cc. of saturated ammonium oxalate are added. In adding the oxalate care is taken that it drops directly into the solution and does not touch the lip of the tube, from which removal by the subsequent washing is likely to be incomplete. The mixture is stirred with a thin footed glass rod. A few drops of 1:1 ammonia are added until the resulting color matches that of a similar volume of a phosphate buffer solution of pH 5.0, containing the same number of drops of indicator. Shohl (1922) has shown that at pH above 4.0 calcium oxalate is completely precipitated. The stirring rod is washed off with a few drops of water. The tube is covered, and the mixture is allowed to stand overnight to complete precipitation.

After precipitation the solution is rapidly centrifuged, and the supernatant fluid is slowly and carefully sucked off without disturbing the precipitate. For this purpose we employ the familiar device of an upturned capillary. A piece of thin glass tubing 2 mm. in diameter is drawn out to a capillary with a U bend at the end. The tip of the suction tube is kept below the surface of the solution in the centrifuge tube, to minimize the chance of drawing in crystals still on the surface of the liquid. Solution is withdrawn until only 0.2 or 0.3 cc. is left in the centrifuge tubes. The tube is then washed twice by centrifugation with 3 cc. portions of the 2 per cent ammonia water. In each washing the ammonia is poured gently down the walls so that the latter are washed about their entire circumference with least possible disturbance of the precipitate. The mixture is then centrifuged and the liquid is drawn off as outlined above.

Resolution of Precipitate and Transfer to Chamber of Manometric Apparatus.—2 cc. of 1 N sulfuric acid are run down the wall of the centrifuge tube in such a manner that every portion of the wall is washed. The tube is dipped into hot water to accelerate resolution of the crystals, then cooled to room temperature.

The outside rim of the centrifuge tube is smeared with a thin film of vaseline, to prevent the solution from creeping over the rim when decanted. The tube is then emptied smoothly, without splashing, into the cup of the Van Slyke-Neill chamber, and the solution is drawn down into the chamber. 4 cc. of water are then used, in three portions, to wash the walls of the centrifuge

tube. With the original mother liquors plus a little of the first washing extending down into the chamber to its 2 cc. mark, the washings are collected in the cup above until they reach the 4 cc. mark on the cup. The washings are then run down into the chamber, making 6 cc. of total solution in it.

Liberation and Measurement of CO_2 in the Gas Apparatus.—The dissolved air and trace of CO_2 in the solution are extracted by evacuating the chamber and shaking for 1 or 2 minutes. The extracted gases are ejected according to the technique described by Van Slyke (1927, p. 240), any liquid reaching the cup being allowed to flow back into the chamber. The wall of the cup is washed down with 1 cc. of acidified 0.15 N $KMnO_4$, which is then allowed to run into the chamber. The chamber is evacuated, and, with the mercury at the 50 cc. mark, is shaken for 3 minutes. In this time the oxalic acid is oxidized to CO_2 and the latter is extracted from solution. The precipitate of partly reduced manganese oxide which first forms may entirely disappear during the last minute, leaving a water-clear solution because of the reducing effect of the mercury in the chamber.

After the CO_2 is extracted the mercury is allowed to ascend in the chamber, with the precautions outlined on p. 533 of Van Slyke and Neill's (1924) paper under "Adjustment of Gas Volume" in CO_2 determinations. If the sample represents 1 cc. or less of serum (0.1 mg. or less of Ca), the gas volume is brought to 0.5 cc. for the p_1 reading. If the sample represents 2 cc. or more of serum (or over 0.2 mg. of Ca), it is preferable to read p_1 with the gas at 2.0 cc. volume.

After the p_1 reading is recorded the cock leading to the leveling bulb is opened, and the bulb is placed at a level slightly below the gas chamber, so that gas in the latter is under slight negative pressure. 1 cc. of 5 N sodium hydroxide, followed by a little mercury, is then admitted to the chamber to absorb the CO_2. The p_2 reading is finally taken with the same gas volume in the chamber as at the p_1 reading.

Determination of c Correction.—A blank analysis is performed in which 2 cc. of 1 N sulfuric acid and 4 cc. of water are placed in the Van Slyke-Neill chamber and analyzed as described above. The $p_1 - p_2$ difference obtained is the c correction for CO_2 from the

TABLE I.

Factors by Which Millimeters P_{CO_2} Are Multiplied to Calculate Oxalic Acid or Calcium.

In all cases it is assumed that the volume, S, of solution extracted in the Van Slyke-Neill chamber is 7.0 cc., and the volume of the chamber 50 cc.

Temperature.	Factors to give mg. Ca in sample analyzed.		Factors to give mg. Ca per 100 cc. in solution analyzed, when sample represents 1 cc.		Factors to give m.-eq. Ca or oxalic acid per liter, when sample represents 1 cc.		Factors to give mM Ca or oxalic acid per liter, when sample represents 1 cc.	
	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.	a = 0.5 cc.	a = 2.0 cc.
°C.								
10	0 000715	0.002804	0.0716	0.2804	0.0357	0.1399	0.01785	0.0700
11	09	2780	09	0.2779	54	87	70	694
12	03	56	03	55	51	75	55	88
13	697	34	697	35	48	65	40	83
14	92	13	91	13	45	54	25	77
15	86	2693	86	0.2691	43	43	13	72
16	81	71	81	71	40	33	00	67
17	76	51	76	50	37	23	0.01688	62
18	71	31	71	32	35	14	76	57
19	66	13	66	14	33	05	64	53
20	62	2595	61	0.2596	30	0.1296	52	48
21	57	78	57	78	28	87	40	44
22	53	61	53	60	26	78	28	39
23	48	43	49	43	24	70	18	35
24	44	26	44	26	21	61	07	31
25	40	10	39	09	19	52	0.01597	26
26	36	2493	35	0 2493	17	44	87	22
27	32	77	31	77	15	36	77	18
28	28	63	27	63	13	29	67	15
29	24	48	24	49	12	22	58	11
30	20	34	21	35	10	15	50	08
31	17	21	18	21	08	08	40	04
32	14	08	15	07	07	02	33	01
33	10	2394	11	0.2394	05	0.1195	25	0.0598
34	07	81	07	82	03	89	15	95

For samples other than 1 cc. the factors in the last six columns are divided by the volume of the sample in cc.

For values of a other than 0.500 or 2.000 cc., the factors in this table are multiplied by $\frac{a}{0.500}$ or $\frac{a}{2.000}$.

reagents. The c value is ordinarily about 5 mm. when the gas is measured at 0.5 cc. volume, 1 or 2 mm. at 2 cc. volume.

Calculation.—The pressure P_{CO_2} due to CO_2 from oxalic acid is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

The P_{CO_2} value thus obtained is multiplied by the proper factor in Table I to estimate calcium. Table I has been computed from the CO_2 factors of Van Slyke and Sendroy (1927), on the assumption that each molecule of oxalic acid yields 99.4 per cent of 2 molecules of CO_2 under the conditions of analysis, as indicated by the experimental work below.

Cleaning Chamber of Manometric Apparatus.—After each analysis the apparatus is cleaned, in the manner described on p. 534 of Van Slyke and Neill's (1924) paper, except that no lactic acid is used. The chamber is washed first with water, then with 1 N sulfuric acid. It is important for this analysis that the chamber be clean and free of organic matter. Lactic acid is oxidized to CO_2 by permanganate; hence it is essential that no traces of it shall be present.

EXPERIMENTAL.

Standardization of Oxalic Acid Solutions.

Standard solutions of oxalic acid were prepared and checked by the following methods of analysis.

(*a*) Titration with sodium hydroxide standardized against 0.1 N hydrochloric acid made from Hulett and Bonner's (1909) constant boiling HCl, the accuracy of which was checked by gravimetric silver chloride analyses. The indicator used was phenolphthalein.

(*b*) Precipitation as calcium oxalate in a solution acidified with acetic acid. The precipitated calcium oxalate was washed and then ignited to CaO and weighed as such. This is the standard gravimetric method.

(*c*) Gravimetric determination of the CO_2 formed by oxidizing the oxalic acid with permanganate. The apparatus previously described by the authors (1927) for the gravimetric determination of CO_2 was used. The oxalic acid with H_2SO_4 was put into the

reaction flask and heated, while an excess of permanganate solution was allowed to drip from the funnel. The CO_2 evolved was collected and weighed in soda-lime U-tubes, as described (Van Slyke and Sendroy, 1927).

In solutions of different lots of acid closely agreeing results by all three methods indicated oxalic acid contents varying from 98.9 to 99.3 per cent of those calculated from the weights of $H_2C_2O_4 \cdot H_2O$ used in preparing the solutions.

Gasometric Determination of CO_2 from Standard Oxalic Acid Solutions.

Of oxalic acid solutions standardized as described above, portions of 1.000 or 2.000 cc. were pipetted into the Van Slyke-Neill apparatus and brought to 6.0 cc. volume by addition of 2 cc. of 1 N sulfuric acid and water. Permanganate was then added and the analysis was completed as described above for serum determinations. The results are given in Table II. They show that under the conditions employed the yield of CO_2 is 99.4 per cent of theoretical. Apparently the oxidation at room temperature does not go quite to completion, but the yield of CO_2 is so nearly quantitative and so constant that the slight shortage can be allowed for in calculations without introducing an error. In computing all the factors of Table I we have accordingly multiplied by 1.006 the theoretical ratio of oxalic acid or calcium to the CO_2 formed by permanganate oxidation.

In the experiments of 5-31-27 and 6-2-27 $MnSO_4$ was added to the $KMnO_4$ to catalyze the reaction if possible. No perceptible difference in the yields was noticed. In the experiments of 7-25-27, and 7-26-27, the samples were first pipetted into 15 cc. centrifuge tubes and washed into the cup of the apparatus as outlined in the description of the method. This check showed the washing to be quantitative. In the experiment of 2-16-29 the solution analyzed was made by dissolving in 2 N sulfuric acid weighed amounts of calcium oxalate. The latter had been precipitated by addition of oxalic acid to an excess of thrice washed calcium carbonate dissolved in HCl and acetic acid. The precipitated calcium oxalate was washed twelve to fifteen times, until the washings were free from calcium and chloride and oxalate, then dried at 60° in a vacuum oven to constant weight. This gave quite an analytically

pure oxalate salt. The results were not noticeably different from those obtained with oxalic acid and sodium oxalate.

Effect of Technique for Washing Calcium Oxalate Precipitate.

Table III illustrates the extent of loss that can occur when the calcium oxalate precipitate is washed by repeated centrifugation,

TABLE II.

Yield of CO_2 from Oxalic Acid in Manometric Apparatus.

Date.	No. of analyses.	Volume of Van Slyke-Neill chamber.	Volume of gas at which P_{CO_2} was observed.	Volume of oxalic acid solution taken.	$H_2C_2O_4$ present according to previous titration and gravimetric analyses.	CO_2 found.	Ratio $\frac{\text{m.-eq. } H_2C_2O_4}{\text{mM } CO_2}$.	Deviation of ratio from average.
		cc.	*cc.*	*cc.*	*m.-eq. per l.*	*mM per l.*		
10- 4-26	4	100	4.0	2	49.65	49.40	1 005	−0 001
10-12-26	2	100	4.0	2	49.65	49.22	1 009	+0.003
10-15-26	5	50	2.0	1	49.65	49 25	1.008	+0.002
10-16-26	4	50	2.0	1	49.65	49.29	1.007	+0.001
11-27-26	12	100	4.0	2	49.65	49 26	1.008	+0.002
3- 1-27	7	50	2.0	1	49.65	48.95	1.014	+0.008
3- 3-27	9	50	2.0	1	49.65	49.16	1.011	+0.005
3- 4-27	10	50	2.0	1	39.84	39.83	1.000	−0.006
3- 8-27	5	50	2 0	1	39 84	39.94	0.998	−0.008
3- 9-27	5	50	2 0	1	39.84	39.79	1.001	−0.005
3-11-27	6	100	4.0	2	39.84	39.94	0.998	−0.008
3-12-27	9	50	0.5	1	4 98	4.96	1.004	−0.002
5-31-27	4	50	2.0	2	7 97	7.94	1.004	−0 002
·6- 2-27	5	50	2.0	2	7.97	7.86	1.014	+0.008
7-25-27	6	50	2.0	1	9.96	9 86	1 010	+0.004
7-26-27	6	50	0.5	1	9 96	9.85	1.011	+0.005
3-23-28	3	50	0.5	1	4 98	4.97	1.002	+0.004
3-23-28	3	50	0.5	1	4.98	4 96	1.004	−0.002
2-16-29	3	50	0.5	2	5.00	4.98	1.002	−0.002
Average..........							1.006	±0.004

especially if the precipitate is stirred with each successive portion of washing solution. The usual ammonia water was used for these washings. The latter in the *a* series were carried out as described above for routine serum analysis, with minimum dis-

turbance of the precipitate, while in the *b* series the precipitate was stirred up with each portion of washing fluid.

Interference of Serum Proteins with Complete Precipitation of Calcium Oxalate.

Calcium was determined in each of 5 sera by two procedures.

(*a*) The analysis was carried out as described above, with preliminary removal of the proteins by means of trichloroacetic acid. Portions of 10 cc. of filtrate, equivalent to 2 cc. of serum, were used for the analyses.

TABLE III.

Effect of Repeated Washing of Calcium Oxalate Precipitate, (a) with and (b) without Stirring Analyses of Known Calcium Solution.

No of washings with 3 cc portions of 2 per cent ammonia	Calcium per 100 cc		Error
	Present	Found.	
	mg.	*mg.*	*per cent*
(a) 3	10 02	10 13	+1 1
(b) 3		9 78	−2 4
(a) 6		9 91	−1 1
(b) 6		8 79	−12 3
(a) 9		9 78	−2 4
(b) 9		7 64	−23 8
(a) 12		9 68	−3 4
(b) 12		6 09	−30 7

(*b*) The calçium oxalate was precipitated in the presence of the serum proteins. In each analysis 2 cc. of serum in a 15 cc. centrifuge tube were diluted to 5 cc. 1 cc. of ammonium oxalate solution was added, and the mixture was left overnight to precipitate. The washing of the precipitate and subsequent steps of the analyses were all carried out according to the routine procedure, except the blank for determination of the *c* correction. For the blank a sample of serum was treated in every way as in the main analysis, except that in place of 1 cc. of ammonium oxalate 1 cc. of 2 per cent NaCl solution was added. The salt solution redissolved globulin that precipitated when the serum was diluted. The p_1 −

p_2 pressure reading obtained in this blank analysis included not only CO_2 from the reagents, but also a slight amount from oxidation of traces of serum organic matter which adhered to the walls of the centrifuge tube even after the two washings with ammonia. Such organic matter was sufficient to increase the value by 1 to 5 mm., when the gas volume was read at 0.5 cc.

That the low results of precipitation in the presence of the serum proteins (fourth column of Table IV) are due to incomplete precipitation of calcium oxalate was shown as follows: A 20 cc. sample of each serum was placed in a large centrifuge tube, 30 cc. of water were added, then 10 cc. of saturated ammonium oxalate

TABLE IV.

Comparison of Serum Calcium Precipitation Performed with and without Preliminary Removal of Proteins.

Analysis No.	Species.	Ca pptd. in trichloroacetic acid filtrate.	Ca pptd. in diluted serum. A	Ca pptd. in filtrate from A after removal of proteins. B	Total Ca obtained in diluted serum and in filtrate. A + B
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	Horse.	13 90	12.96	0.95	13.91
2	Ox.	10.67	10.13	0.51	10.64
3	Man	7.01	6 25	0.75	7 00
4	Ox.	11.29	9 73	1.66	11.39
5	"	10.64	9.95	1.21	11 16

solution. The calcium oxalate was allowed to precipitate overnight. The mixture was then centrifuged. The supernatant diluted serum was poured through an ashless filter into a 100 cc. volumetric flask. The transfer was made more nearly quantitative by washing the filter. To the filtrate 20 cc. of 20 per cent trichloroacetic acid were added, the mixture was further diluted to 100 cc., let stand a half hour, and then filtered from the protein coagulum. 10 cc. portions of the protein-free filtrate were transferred to centrifuge tubes and brought with sodium acetate and ammonia to pH 5.0, as in the routine method described above.

A precipitate of calcium oxalate formed, proving that the preceding calcium precipitation in the presence of the serum proteins had been incomplete.

The precipitate was washed and determined gasometrically as described for the routine procedure. The amounts of extra calcium recovered are shown in the fifth column of Table IV. Added to the amounts obtained in the first precipitate they yield, except in Analysis 5, total calcium values as near as could be expected to those obtained by the routine single precipitation with preliminary removal of proteins (compare third and last columns).

Micro Analyses of Standard Calcium Solutions by Gasometric and Titrimetric Methods.

As stated above, the precision of micro calcium methods rests upon details, of which the most important appears to be the technique of washing the precipitate. Before we settled upon the procedure described above for the routine method, we performed some 600 gasometric and titrimetric analyses under varying conditions of precipitation and washing. A few illustrative results are given in Table V.

The standard calcium solutions were made from reprecipitated calcium carbonate which was thoroughly washed and finally dried at 280°. Standard solutions were made from weighed portions, which were dissolved with a slight excess of hydrochloric acid. The accuracy of the standard solutions was controlled by gravimetric macro analyses, in which the calcium was precipitated as oxalate and weighed as ignited CaO in platinum crucibles.

The micro gasometric and volumetric analyses were carried out in triplicate on solutions containing, as does blood serum, between 8 and 10 mg. of calcium per 100 cc. The samples taken varied between 1 and 5 cc. The addition of ammonium oxalate was carried out as in the routine procedure previously outlined. A series of selected but representative results is given in Table V. The time allowed for precipitation ranged from 1 to 24 hours. The washings were made with portions of the 2 per cent ammonia water which were varied in number and volume.

The procedure finally adopted for routine serum calcium determinations, in which the washing is carried out with two portions of 3 cc. each of the dilute ammonia, was found to give the best results. With this technique the micro analyses showed maximum deviations of ± 3 per cent from the amount of calcium present, plus and minus deviations being about equally frequent. The

TABLE V.

Gasometric and Titrimetric Analyses of Solutions Containing Known Amounts of Calcium.

Date.	Sample.	Time of precipitation.	Precipitate washed with 2 per cent ammonia.	Ca per 100 cc.			Error.	
				Present.	Found.		Gasometric.	Titrimetric.
					Gasometric.	Titrimetric.		
	cc.	*hrs.*	*cc.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
2-25-26	3	20	3 × 3.5	10 02	10.02	10.10	0.0	+0.8
3- 4-26	3	20	3 × 3.5	10 02	10 02	10.06	0.0	+0 4
3-21-27	1	20	2 × 4	10.02	9 74	9.78	−2.8	−2.4
	2		2 × 4	10.02	9.83	9.73	−1.9	−2.9
	3		2 × 4	10.02	9 79	9.88	−2 2	−1 4
	5		2 × 4	10.02	9.77	9 85	−2 5	−1.7
4- 2-27	1	24	2 × 4	10.02	9.95	10.22	−0.7	+2.0
	2		2 × 4	10.02	10.04	10.17	+0.2	+1.5
	3		2 × 4	10.02	9.99	10.17	−0 3	+1.5
	5		2 × 4	10.02	9.99	9.91	−0 3	−1.1
7-19-27	2	4	2 × 8	8.66	8.51	8.52	−1.7	−1.6
	2		3 × 3	8.66	8.43	8.62	−2.5	−0 5
7-20-27	3	3	2 × 5	8.66	8.74	8.70	+0.9	+0.5
	3		2 × 8	8.66	8.65	8.70	0.0	+0.5
1-21-27	2	1	2 × 6	8.66	8.51	8.63	−1.7	−0.3
	3		2 × 6	8.66	8.53	8.43	−1.5	−2.7
7-25-27	5	1	2 × 3	8.66	8.61	8.65	−0.6	−0.1
	5		2 × 6	8.66	8.44	8.49	−2.5	−2.0
7-27-27	1	20	2 × 3	8.66	8.91	9.03	+2.9	+4.3
	2		2 × 3	8.66	8.59	8.64	−0.8	−0.2
3- 5-28	1	20	2 × 3	8.61	8.68		+0.8	
	2		2 × 3	8.61	8.50		−1.3	
	1		2 × 3	10.02	10.25		+2.3	
	2		2 × 3	10.02	10.14		+1.2	

great majority of deviations were within ±1 per cent of the amount of calcium present.

Whatever washing technique was employed, titrimetric and gasometric analyses of precipitates similarly handled usually agreed within ±1 per cent, indicating that with the amounts of

TABLE VI.

Comparison of Gasometric and Titrimetric Analyses of Serum.

Date.	Serum.	Volume of serum represented in sample.	Ca per 100 cc.		Percentage difference of titrimetric from gasometric.
			Gasometric.	Titrimetric.	
		cc.	mg.	mg.	
5- 6-29	Ox.	2	11 55	10.93	−5.4
		1	11.45	11.41	−0.4
3-26-29	Horse.	2	13.91	13.88	−0 2
3- 9-29	Ox.	2	10.60	10.74	+1.3
1-28-29	Man.	2	7.01	7.02	+0.1
1-22-29	Ox.	2	11.16	11.42	+2 3
1-17-29	"	2	10.70	11 01	+2.9
			10 56	10.67	+1.0
1- 9-29	"	2	11.19	10.98	−1.9
			10.86	10.94	+0.7
12-31-28	"	2	10.41	10.72	+3.6
		2	10.46	10.67	+2.6
3-23-28	Horse.	2	13.93	13 37	−4.0
3-20-28	Ox.	2	10.76	11 11	+3 3
6-22-27	Man.	2	10 08	10 18	+1.0
6-11-27	"	2	5.52	5 72	+3.6
		1.4	8.18	8 31	+1.6
		1	5.79	5.92	+2.2
		2	9 53	9.87	+3 6
6- 8-27	"	2	9 52	9.78	+2.7
		1.6	6.89	7.03	+2.0
5-20-27	Horse.	2	11 59	11.62	+0 3
5-17-27	"	2	11 68	11.71	+0 3

material used, the error involved in the final analysis by either method is unimportant compared with the errors involved in prior handling of the calcium oxalate precipitate.

Comparison of Gasometric and Titrimetric Calcium Determinations in Blood Serum.

The determinations were carried out according to the method described in this paper, except that in the titrimetric analyses the

final solution of calcium oxalate, instead of being cooled and transferred to the Van Slyke-Neill apparatus, was titrated hot in its centrifuge tube with 0.01 N or 0.005 N permanganate. The results of a series of parallel analyses are given in Table VI.

SUMMARY.

A method is described, whereby oxalic acid is quantitatively estimated by oxidation with an excess of permanganate in the Van Slyke-Neill apparatus, the evolved CO_2 being measured manometrically.

Applications of the method to calcium determinations in general, and to serum micro calcium determinations in particular, have been developed.

In connection with the latter it has been shown that removal of the serum proteins is a necessary preliminary to complete precipitation of calcium as oxalate.

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THE DETERMINATION OF "FREE NICOTINE" IN TOBACCO: THE APPARENT DISSOCIATION CONSTANTS OF NICOTINE.*

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The nicotine content of different samples of cured tobacco usually varies between 1 and 3.5 per cent of the dry weight and, since nicotine is a relatively strong basic substance, it might be supposed that the hydrogen ion concentration of extracts of tobacco would run parallel with the nicotine content, the reaction of an extract derived from a tobacco containing a high proportion of nicotine being more alkaline than one from a tobacco of low nicotine content. Data secured by Bailey, Nolan, and Mathis (1) (see Table IV) indicate that this is not the case; no simple relation is apparent between the total nicotine content and the reaction of the extract of different samples. A marked parallelism was observed, however, between the proportion of so called "free nicotine" and the reaction of the extract, high proportions of free nicotine being invariably found in tobaccos yielding an alkaline extract, while low proportions are found in tobaccos yielding an acid extract. Free nicotine is determined by subjecting a sample of tobacco to steam distillation without the addition of alkali (2), and its estimation is of some importance since the harsh flavor of certain tobaccos is attributed to the presence of a high proportion of this component.

The relationship between the reaction of the extract and the estimated proportion of free nicotine in a sample of tobacco

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indicated that the free nicotine which is observed owes its origin to the hydrolysis of nicotine salts. The equation

$$pH = pK + \log \frac{\text{(free base)}}{\text{(salt of base)}}$$

shows that the degree of hydrolysis, represented by the last term, depends on the reaction of the solution and the dissociation constant of the base. Once the magnitude of the dissociation constant of nicotine has been ascertained, a curve can be constructed from which the degree of hydrolysis of the nicotine salt can be evaluated. Consequently the proportion of the total nicotine which is present in the free form in a sample of tobacco can be read directly from this curve at the point corresponding to the reaction of an extract of the sample.

TABLE I

Apparent Dissociation Constants of Nicotine

	Authors 20°	Kolthoff, about 16°
pK'_{a_1}	3 22	3 2
pK'_{a_2}	8 11	8 0

Nicotine is a di-acid base and its apparent dissociation constants have been determined by Kolthoff (3). Since he employed a somewhat impure preparation of the substance and determined only three or four points on each limb of the curve by a colorimetric method, it was thought desirable to repeat the determination upon highly purified material by the electrometric method. The values of the apparent dissociation constants we have obtained are given in Table I, together with those of Kolthoff. Our values are calculated from Equation 31 of Van Slyke (4) and from the Henderson-Hasselbalch equation respectively and are not corrected for the activity of the ions. Kolthoff calculated his value for pK'_{a_1} making the arbitrary assumption of an activity coefficient of 0.8 for the ions of the nicotine salt. His value for pK'_{a_2} was not corrected for activity. The data from which our values for the constants have been calculated are shown in Tables II and III and the figures in Table I are the average of the results of all save the first and last observations. The titration curve is

shown in Fig. 1 and a curve from which the proportion of free nicotine in a tobacco extract of known reaction may be read is shown in Fig. 2.

Preparation of Pure Nicotine.—A hot water extract of fresh green tobacco leaves was concentrated *in vacuo* and, after the

TABLE II

First Apparent Dissociation Constant of Nicotine at 20 ± 0.5°.

$$pK'_a = pH - \log \frac{B + H^+}{C - (B + H^+)}\ ^*$$

NaOH (b)	Normality. (H^+)	$0.000997 \times (b) = B$	$B + H^+$	$\frac{0.1059 \times 24.97}{100} = C$	$C - (B + H^+)$	$\log \frac{B + H^+}{C - (B + H^+)}$	pH	pK'_a
cc								
0 1	0 004	0 00010	0 0041	0 02645	0 0224	−0 74	2 40	3 1
2 1	0 0027	0 00209	0 00479	0 02645	0 0217	−0 66	2 57	3 2
3 1	0 0020	0 00309	0 00509	0 02645	0 0214	−0 62	2 69	3 3
8 1	0 0011	0 00808	0 00918	0 02645	0 0173	−0 28	2 95	3 2
13 1	0 00056	0 0131	0 01366	0 02645	0 0128	+0 03	3 25	3 2
18 1	0 00026	0 0180	0 01826	0 02645	0 00819	+0 35	3 58	3 2
20 1	0 00020	0 0201	0 02030	0 02645	0 00615	+0 52	3 70	3 1
22 1	0 00013	0 0220	0 02213	0 02645	0 00432	+0 71	3 89	3 1
23 1	0 000081	0 0231	0 02318	0 02645	0 00327	+0 85	4 09	3 2
24 1	0 000063	0 0240	0 02406	0 02645	0 00239	+1 00	4 20	3 2
25 1	0 000035	0 0250	0 02504	0 02645	0 00141	+1 25	4 45	3 2
25 6	0 000020	0 0255	0 02552	0 02645	0 00093	+1 44	4 69	3 2
26 1	0 0000085	0 0260	0 02601	0 02645	0 00044	+1 77	5 07	3 3
Average								3 2
Kolthoff								3 2

B = molar concentration of added base. C = molar concentration of nicotine dihydrochloride.

* Van Slyke, Equation 31 (4).

addition of an excess of sodium hydroxide, was subjected to steam distillation. The distillate was collected in hot water containing in suspension approximately enough picric acid to form the dipicrate with the previously determined total quantity of nicotine in the extract. The suspension was continually stirred. Distillation was continued until practically all of the nicotine had

been driven over and, in order to make sure that the nicotine was completely converted to dipicrate, the distillate was, if necessary, then treated with a hot saturated aqueous picric acid solution until a sample gave a trace of precipitate of nicotine dipicrate when tested with a dilute solution of nicotine. The distillate was cooled and the crude nicotine dipicrate was filtered off and washed

TABLE III.

Second Apparent Dissociation Constant of Nicotine at 20 ± 0.5°.

$$pK'_a \quad pH - \log \frac{B}{C - B}$$

NaOH (b)	(b) − 26.5 cc. excess NaOH = (c)	0.000997 × (c) = B	$\frac{0.1059 \times 24.97}{100}$ = C	C − B	log $\frac{B}{C - B}$	pH	pK'_{a_2}
cc.							
26.6	0.1	0.00010	0.02645	0.0264	−2.42	5.87	8.29
27.1	0.6	0.000598	0.02645	0.0259	−1.64	6.46	8.10
27.6	1.1	0.00110	0.02645	0.0254	−1.36	6.72	8.08
28.1	1 6	0.00159	0.02645	0.0249	−1.10	6.98	8.08
30.1	3.5	0.00349	0.02645	0.0230	−0.82	7.31	8.13
33.1	6.6	0.00658	0.02645	0.0199	−0.48	7.64	8.12
38.1	11.6	0.0116	0.02645	0.0149	−0.11	8.02	8.13
43.1	16.6	0.0166	0.02645	0.00985	+0.23	8.36	8.13
48.1	21.[illegible]	0.0215	0.02645	0.00495	+0.64	8.77	8.13
50.1	23.6	0.0235	0.02645	0.00295	+0.90	9.00	8.10
52.1	25.6	0.0255	0.02645	0.00095	+1.43	9.34	7.91
Average							8.11
Kolthoff							8.0

B = molar concentration of added base. C = molar concentration of nicotine monohydrochloride.

with water and alcohol. This material decomposed at 218–220° (short stem thermometer). It was recrystallized twice from large volumes of boiling water and dried at 105.° It then decomposed at 224–226°, and contained 17.89 per cent of nitrogen (theory 18.04 per cent) and 73.35 per cent of picric acid (theory 73.86 per cent).

The recrystallized dipicrate was suspended in a 20 per cent

excess of 20 per cent hydrochloric acid and heated on the steam bath for several hours. It was then chilled and the picric acid was filtered off on a fritted glass funnel and washed free from nicotine with 20 per cent hydrochloric acid. The filtrate was freed from picric acid by extraction with ether, was diluted with 2 volumes of water, boiled with norit until colorless, and was then concentrated to a sirup. The sirup was diluted with several volumes of water and an excess of sodium hydroxide was added to the carefully cooled solution. The free nicotine was extracted with ether, the ether solution was dried over sodium sulfate, and the ether was evaporated. The pale yellow oil remaining was distilled *in vacuo*, the fraction boiling at 87° at 8 mm. pressure being collected.

A tobacco extract containing 62 gm. of nicotine as indicated by the silicotungstic acid method gave 234 gm. of crude dipicrate (theory 243 gm.) and 205 gm. of twice recrystallized dipicrate (84 per cent). This yielded 51 gm. of pure distilled nicotine (82 per cent). Over 95 per cent of the picric acid was recovered. Nicotine dipicrate is soluble to the extent of 0.5 to 0.6 per cent in boiling water and has a solubility of 0.059 gm. in 100 cc. at 22°.

Preparation of Solutions of Nicotine.—Pure nicotine (17.230 gm.) was dissolved in 110 cc. of 1.990 N hydrochloric acid and diluted to 1 liter. The amount of acid was chosen so as to provide a small excess over the amount necessary to form the dihydrochloride. Loss of nicotine from the stock solution as well as absorption of carbon dioxide was thereby prevented. An analysis of the solution by the silicotungstic acid method indicated the presence of 17.12 gm. of nicotine per liter. The average of 17.18 gm. per liter (0.1059 M) was taken for the calculation of the composition of the solutions used for the hydrogen ion concentration measurements.

The solutions were prepared by pipetting 24.97[1] cc. of the stock solution into 100 cc. flasks, and adding to each different quantities ranging from 0 to 2.4 equivalents of 0.09970 M sodium hydroxide solution (CO_2-free) from a calibrated burette. The solutions were then diluted to volume at room temperature (20°). The titration was thus conducted at constant volume.

It is clear that 26.53 cc. of 0.0997 N sodium hydroxide solution are required to liberate one basic group from the nicotine present

[1] The 25 cc. pipette used delivered 24.97 cc. at 20°.

in 24.97 cc. of 0.1059 M stock solution and 53.06 cc. to liberate both groups. Titration of 24.97 cc. of stock solution to methyl red

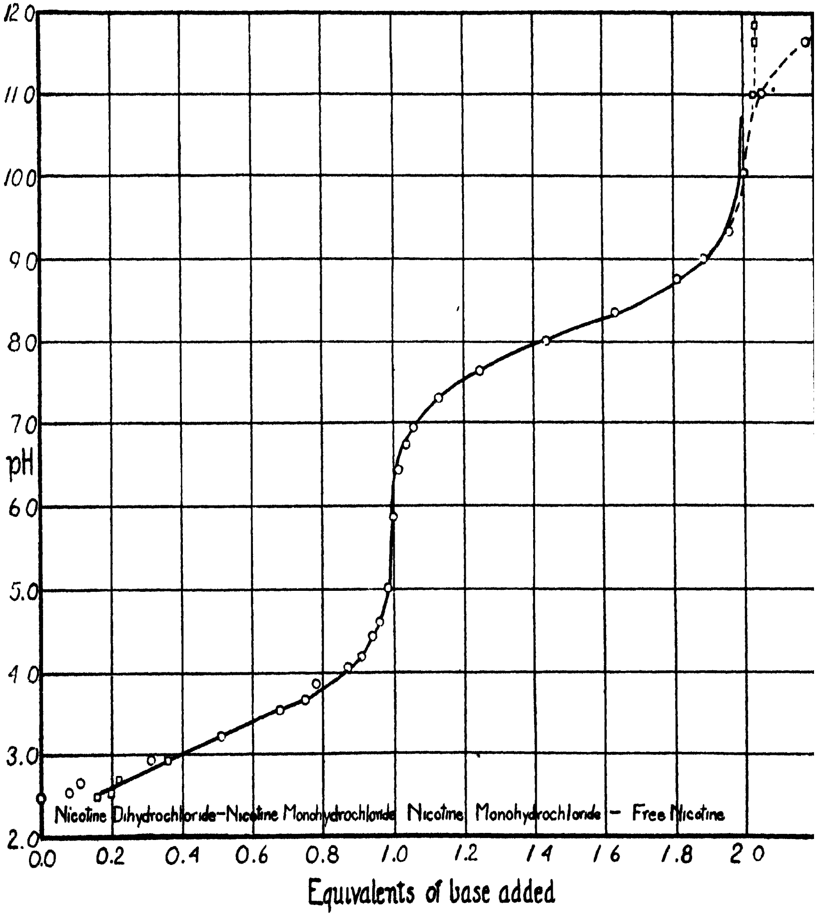

FIG. 1. Titration curve of nicotine dihydrochloride with sodium hydroxide at 20 ± 0.5°. The circles represent experimentally determined points; the curve is calculated from the dissociation constants in Table I.

indicated that this amount contained 1.9 cc. of 0.0997 N hydrochloric acid in excess of the quantity required to form the dihydrochloride of nicotine. This correction was therefore deducted

On page 239, Vol. lxxxiv, No. 1, October, 1929, Fig. 2, ordinate scale, read *9.0, 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5* for *8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, 5.0.*

from the amount of 0.0997 N sodium hydroxide added to each solution when preparing the mixtures for hydrogen ion concentration measurements.

The measurements[2] of the hydrogen ion potential were made in a Clark rocking electrode vessel with 0.1 N hydrochloric acid (pH = 1.08) as the reference standard in conjunction with the

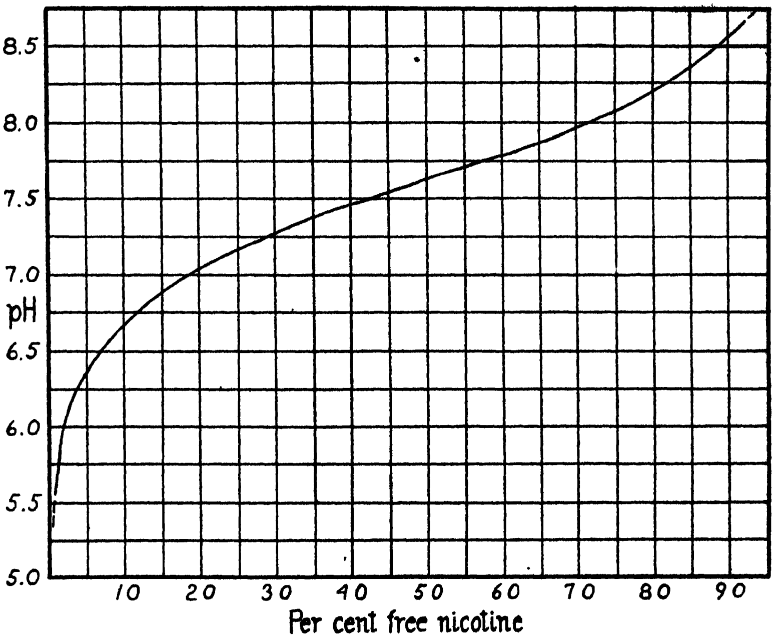

FIG. 2. Titration curve of nicotine, showing proportion of free base at reactions within the range of physiological interest.

saturated calomel half-cell. All measurements were carried out at a temperature of 20 ± 0.5°, and were repeated until agreements within 0.5 millivolts were obtained.

The data are represented by circles in Fig. 1. The solid line curve is calculated from the Henderson-Hasselbalch equation, the

[2] We are indebted to Professor D. M. Hitchcock of the Department of Physiology of the Yale Medical School for the privilege of using his equipment and for much helpful advice.

average values for pK'_{a_1} and pK'_{a_2} from Tables II and III being used. The points indicated by squares represent corrections for the effect of excess H^+ or OH^-.

In Fig. 2 is given a curve showing the relation between nicotine monohydrochloride and free base, plotted on a larger scale over the range of reaction of interest in the determination of the proportion of free nicotine in tobacco. To carry out this determination 2.5 gm. of ground tobacco are suspended in 50 cc. of water and stirred at room temperature for 5 to 10 minutes. After settling, a little of the clear fluid is decanted into the electrode

TABLE IV.

*Proportion of Free Nicotine in Various Samples of Tobacco (Cigarettes and Cigars).**

Sample No.	Nicotine.	pH of extract.	Proportion of total nicotine as free nicotine, from curve in Fig. 2	Proportion of dry weight as free nicotine, from curve in Fig. 2.	Free nicotine by distillation
	per cent		*per cent*	*per cent*	*per cent*
8731	2.30	5 1	>1.0		0.26
8737	1.28	5 2	>1.0		0.15
8742	1.45	5 9	>1 0	>0.015	0.33
8739	1.90	6 8	4 3	0 082	0.60
8740	1 16	7.5	19 5	0.23	0.79
8741	1.31	7.5	19.5	0.26	0.72
689	1.38	7.8	38	0.52	1.11

* Determinations by Bailey, Nolan, and Mathis (1). We wish to thank them for permission to use these data.

vessel and the reaction is determined by the rapid quinhydrone electrode technique. A total nicotine determination is made on another sample. The proportion of the total nicotine present as free nicotine is then read from the curve in Fig. 2 at a point corresponding to the hydrogen ion concentration of the extract of the sample. Data secured in this way are presented in Table IV. The curve in Fig. 2 applies only to the temperature at which the determinations of the dissociation constants were made. Free nicotine determined as is here suggested therefore represents only the proportion free at this temperature and there is no reason to suppose that the magnitude of the results should duplicate those secured by distillation with steam. By this method, however, a

factor of rather precise physicochemical meaning is secured which should prove equally as useful in forming a judgment of the quality of tobacco as the result of the distillation method.

SUMMARY.

When samples of tobacco are subjected to steam distillation without the addition of alkali, a part of the nicotine usually passes over into the distillate; it has been noted that the relative magnitude of this part varies with the hydrogen ion concentration of the extract. The determination of the free nicotine, as this volatile part of the nicotine has been designated, is of some importance in the chemical examination of tobacco, since the harsh flavor of certain tobaccos has been attributed to a high proportion of this component. Free nicotine is present in tobacco because of the hydrolysis of the nicotine salts in the tissue. The proportion present at ordinary temperatures may therefore be read directly from the dissociation curve of nicotine at the point corresponding to the reaction of the sample. The apparent dissociation constants of highly purified nicotine have been determined by the electrometric method and a dissociation curve is given from which the proportion of the total nicotine occurring in the free form in a tobacco extract of known hydrogen ion concentration may be read. It is suggested that this method for the estimation of the free nicotine of tobacco is simpler and more precise than that hitherto employed.

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COMPARISON OF RATES OF SUGAR DISAPPEARANCE AND CARBON DIOXIDE FORMATION DURING FERMENTATION OF GLUCOSE.

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In an investigation of the amount of sugar in urine (Van Slyke and Hawkins, 1929) we noticed that it was impossible to detect any glucose by the reduction method after the glucose-yeast mixture had stood for 15 minutes, whereas it was necessary to allow the mixture to stand 1 hour in order to get 80 per cent of the theoretical yield of carbon dioxide from the glucose. This suggested that the combination or absorption of glucose by the yeast is a rapid reaction, while the breaking down of glucose into alcohol and carbon dioxide is a much slower one.

Slator (1906) explains the mechanism of fermentation on the hypothesis that sugar diffuses into the yeast cell and combines with the enzyme, and that this compound decomposes either directly or indirectly into alcohol and carbon dioxide with regeneration of enzyme and immediate formation of more compound with sugar. In this series of reactions, he states that the decomposition of the compound is the one which proceeds slowest and is therefore the most important in determining the velocity reaction. He found that when the concentration of the sugar was above 1 per cent it had no influence on the velocity reaction, but that the concentration of the sugar would have an influence if the concentrations of sugar and yeast were such that an appreciable amount of enzyme was left uncombined.

We have measured simultaneously, by observing the disappearance of reducing material from solution, the rate at which glucose combines with the yeast and also, by observing the rate of CO_2 formation in the yeast-sugar solution mixture, the rate at which the sugar breaks down into alcohol and carbon dioxide.

EXPERIMENTAL.

50 cc. of a standard glucose solution were mixed with 50 cc. of yeast suspension and 15 cc. portions of this mixture were run rapidly into 20 cc. tubes filled with mercury and connected at the bottom with mercury leveling bulbs. The tubes used were of the type marked *J* in Fig. 3 of Austin, Cullen, *et al.* (1922). Each tube was sealed by clamping the rubber inlet tube at the top with a screw clamp. At various time intervals after the mixing of the yeast and glucose, 1 cc. of a 4 per cent solution of sodium fluoride in 8 per cent sodium hydroxide was run into each tube in order to kill the yeast and prevent the escape of CO_2. The tubes were shaken vigorously to insure thorough mixing.

Determination of Carbon Dioxide.

3 cc. samples of the alkali-treated suspension of yeast in glucose solution were transferred by means of a rubber-tipped, stop-cock pipette to the chamber of Van Slyke and Neill's manometric apparatus and analyzed for CO_2 as described by them (1924). The CO_2 factors of Van Slyke and Sendroy for the manometric blood gas apparatus (1927) were used to calculate the CO_2. Controls were run at the same time by substituting distilled water for the glucose solution in order to correct for the CO_2 present in the yeast itself.

Determination of Glucose by Ferricyanide Reduction.

5 cc. portions of the alkali-treated yeast-glucose solutions were mixed with 10 cc. of distilled water, 5 cc. of 0.2 N H_2SO_4, and 1.5 gm. of Lloyd's reagent. The mixture was shaken for 1 minute and filtered through a dry filter paper in order to obtain a clear filtrate. The sugar remaining in the filtrate was then determined by the Van Slyke-Hawkins gasometric blood sugar method (1928).

Control analyses in which the sugar solutions were replaced by water were run at the same time to determine p_0 readings.

Amount of Glucose Removed from Solution Estimated from Decrease in Reducing Power and From Increase in CO_2.

The results of these determinations are given in Table I. They indicate that the initial reaction rate for the removal of glucose

TABLE I.

Amount of Glucose Removed from Solution Estimated from Decrease in Reducing Power and from Formation of CO_2.

Amounts of glucose and yeast in 100 cc. suspension.	Time after addition of yeast to glucose solution.	Series 1.				Series 2.				Series 3.			
		Decrease in glucose determined by ferricyanide reduction method. *a*	CO_2 formed. *b*	Glucose fermented calculated from CO_2 formed. *c*	Ratio $\frac{c}{a}$	Decrease in glucose determined by ferricyanide reduction method. *a*	CO_2 formed. *b*	Glucose fermented calculated from CO_2 formed. *c*	Ratio $\frac{c}{a}$	Decrease in glucose determined by ferricyanide reduction method. *a*	CO_2 formed. *b*	Glucose fermented calculated from CO_2 formed. *c*	Ratio $\frac{c}{a}$
	min.	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*	
Mixture 1.	5	120	18.6	38	0.32	98	13.2	27	0.28		40.0	82	
250 mg. glucose and 10	10	203	64.5	132	0.65		38.6	79		226	87.0	178	0.79
gm. yeast.	15	250	84.0	172	0.69		57.7	118		245	99.2	203	0.83
	30	250	90.8	186	0.74	250	88.9	182	0.73	250	110.4	226	0.90
	60	250	95.8	196	0.77	250	99.7	204	0.82	250	119.6	245	0.98
Mixture 2.	5	69	24.4	50	0.72	56	18.6	38	0.67	63	22.0	45	0.71
125 mg. glucose and 10	10	119	45.4	93	0.78	107	36.2	74	0.69	118	44.0	90	0.76
gm. yeast.	15	124	47.4	97	0.78	121	43.5	89	0.73	125	44.9	92	0.74
	30	125	48.4	99	0.79	125	45.4	93	0.74	125	46.9	96	0.77
	60	125	49.3	101	0.81	125	49.8	102	0.82	125	47.9	98	0.78
Mixture 3.	5	55	10.2	23	0.42	36	4.4	9	0.25	36	7.8	16	0.44
125 mg. glucose and 5	10	83	25.4	52	0.63	42	10.3	21	0.50	51	15.6	32	0.63
gm. yeast.	15	102	36.6	75	0.73	51	16.1	33	0.65	70	22.9	47	0.67
	30	125	46.9	96	0.77	98	33.2	68	0.69	114	40.1	82	0.72
	60	125	48.4	99	0.79	125	44.0	90	0.72	125	44.5	91	0.73

from solution by yeast is much more rapid than the initial reaction rate for the decomposition of glucose to alcohol and CO_2. The rate during the first 5 minutes at which glucose is decomposed is in Mixtures 1 and 3 only from 0.25 to 0.44 as fast as the rate at which glucose disappears from solution.

In Mixture 2 the ratio of yeast to glucose is evidently so high that the initial lag in CO_2 formation is more nearly overcome in the first 5 minute period. In this mixture the glucose decomposition during the first 5 minutes is 0.67 to 0.72 as much as the glucose removal.

Further evidence of the initial lag in CO_2 formation is seen in the fact that in Mixtures 1 and 3 actually less CO_2 was formed in the first 5 minutes of the reaction than in the second 5 minutes.

From comparison of the results from Mixtures 1 and 2 it is evident that, with a constant amount of yeast present, the rate of glucose disappearance during the first 5 minutes is nearly proportional to the glucose concentration. CO_2 formation, however, does not show in its initial rate any proportionality to glucose concentration.

From comparison of Mixtures 1 and 3 it is evident that, with a constant initial glucose concentration, the initial rates of both glucose disappearance and CO_2 formation increase with the concentration of yeast.

Our method of CO_2 determination measured the total CO_2 content of the yeast-solution mixture, cells as well as fluid. It is consequently impossible to ascribe the observed initial lag in CO_2 formation to retention of CO_2 in the yeast cells.

Mixtures containing glucose, water, and yeast in the same proportions gave in the different series of experiments varying ates of both glucose disappearance and CO_2 formation. This variation is attributable to inconstancy in the activity of the yeast used. Different Fleischmann yeast cakes were used for each day's experiments, and the activity of the yeast in them varied. With all of them, however, the same lag in initial CO_2 formation behind glucose disappearance was observed.

In Table I the amounts of glucose corresponding to the observed amounts of CO_2 formed have been calculated in accordance with the equation $C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$. This equation is not quite exact, as small amounts of other products are formed,

so that the glucose equivalent of CO_2 formed by fermentation, even of indefinite length, is actually slightly greater than corresponds to the equation. However, multiplying all the figures in the last column of figures for each series by a value a few per cent above unity would not change significantly the interpretation of the results with regard to the lag in CO_2 formation. CO_2 formation begins more slowly than does disappearance of reducing sugar, and CO_2 formation is still progressing at an appreciable rate during the last half hour in the first two series, after glucose removal from the solution has become complete.

SUMMARY.

The rate of removal of glucose from solution by yeast and the rate at which glucose decomposes, as indicated by CO_2 formation, have been measured. During the initial stage glucose removal takes place more than twice as rapidly as glucose decomposition.

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THE RELATION OF VITAMIN A CONTENT TO SIZE OF LEAVES.

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Vitamin A is very unequally distributed in the parts of a plant but the leaves contain a large fraction of it. It seems likely that it is formed in the leaves and then carried to other tissues. If the more actively functioning cells elaborate it more rapidly we should expect young leaves to be more potent; if it is elaborated only in the chlorophyll-bearing cells we should expect thin leaves of relatively greater surface area to be the richer.

In 1921 Coward and Drummond (2) concluded that green leaves form large amounts of vitamin A and that leaves deficient in chlorophyll do not synthesize it. In 1925 Coward (1) reported that in her tests on the persistence of vitamin A in plant tissues she had used quantities of leafy tissue with as nearly as possible equal areas of leaf surface rather than with equal weights, because light is a controlling factor in the formation of vitamin A. Dye, Medlock, and Crist (4) and Crist and Dye (3) have given experimental evidence that the vitamin A of plant tissues is associated with its greenness.

Heller (6) germinating seeds in the dark and in light from various sources showed that the quantity of vitamin A synthesized is dependent on the light received, its intensity and wave-length. There is a relation between light and chlorophyll and vitamin A; and the amount of light that leafy tissue receives is dependent on its surface area.

New Zealand spinach plants seemed to offer opportunity for comparison of vitamin A contents of small and larger leaves

because on the same plant may often be found leaves of any size, from those just forming up to others having an upper surface area of 10 to 12 square inches. In general it may be assumed that the larger the leaf the older it is.

It was planned to determine the relative vitamin A contents of equal weights of leaves of three distinctly different sizes, the rat growth method being employed to measure vitamin potency.

Procedure.

Size of Leaf.—Blue-prints were made of a great number of leaves and the surface areas were determined with a planimeter. Celluloid patterns were then cut, as accurately as possible, of leaves with upper surface areas of 4, 5, 8, and 10 square inches. With these it was easy to feed portions of leaves the areas of which were known within definite limits. We used (*a*) leaves less than ½ square inch in area, usually about ¼ inch, designating these small leaves; (*b*) medium leaves between 4 and 5 square inches, except during a few weeks of the many months of the experiment when it was necessary to take smaller ones, but even then larger than 2 square inches; (*c*) leaves between 8 and 10 square inches in area, which are designated in this paper as large leaves.

Weighing of Leaves.—Desired quantities of leafy tissue were carefully and quickly weighed out within less than a half hour after picking, usually within 15 minutes, to lower so far as possible differences in weight due to change in moisture content. From a small leaf a triangular piece was cut out at the base to eliminate the heavier portion of the midrib; from other leaves the portions were cut from along an edge. At first a chainomatic chemical balance with the bar set to indicate the desired mass was used; later, time was saved by using a small, sensitive torsion balance.

During the winter and spring New Zealand spinach grown in the greenhouse was used; during the summer and fall leaves were picked from garden grown plants.

Rats.—Albino rats were used that for generations had been on a grain diet supplemented by milk and occasionally by leafy vegetables. For our stock, Steenbock's diet (10) is modified to contain wheat germ and yeast. The laboratory technique is that of Ferry (5). Litters are reduced to seven animals at 2 days of age.

Method.

When 28 days old the rats were put into individual cages on an irradiated diet consisting of purified (9) casein 18 per cent, dextrinized (7) corn-starch 76 per cent, salt mixture (8) 4 per cent, and agar 2 per cent. A saltspoonful of yeast weighing approximately 0.4 gm. was fed separately each day. Cessation of growth was the chief criterion for adjudging depletion of vitamin A. One rat from each litter was kept as a negative control, the others were fed graded quantities of leaf according to the Sherman-Munsell (9) method for determination of vitamin A. New Zealand spinach is exceedingly rich in vitamin A, so to minimize errors in weighing the small quantities desired, it was in general fed three times per week.

An attempt was made with each size of leaf to find the quantity of New Zealand spinach which, when supplementing a diet adequate in all respects except vitamin A, will promote an increase in weight of 25 gm. in rats whose body stores of vitamin A have been depleted.

Results.

With small leaves somewhat less than 90 mg. and more than 70 mg. per week was required; with medium leaves about 90 mg. were necessary; and with large leaves more than 110 mg. and less than 120 mg. was effective in promoting the desired increase in weight.

As shown in Chart I for leaves of medium size, or in Table I for the three sizes of leaves, the method used, when several animals are employed, is delicate enough to distinguish between the vitamin potencies of quantities of New Zealand spinach differing by only 10 mg. of leafy tissue per week.

Chart II brings out differences in effect upon growth of feeding each week 70 mg. of small leaves or 70 mg. of tissue from medium sized leaves. The difference at the end of the 8 week period is practically the same as that found when 70 mg. and 80 mg. of medium leaves were fed; that is, 70 mg. of small leaves are as potent as 80 mg. of medium leaves, as Chart II also shows. The two groups of rats fed these quantities had average initial weights that differed by an insignificant amount, 0.6 gm.; the increases

in weight of the rats and the food consumptions of the basal diet were equal. When large leaves are fed, more than 110 mg. per

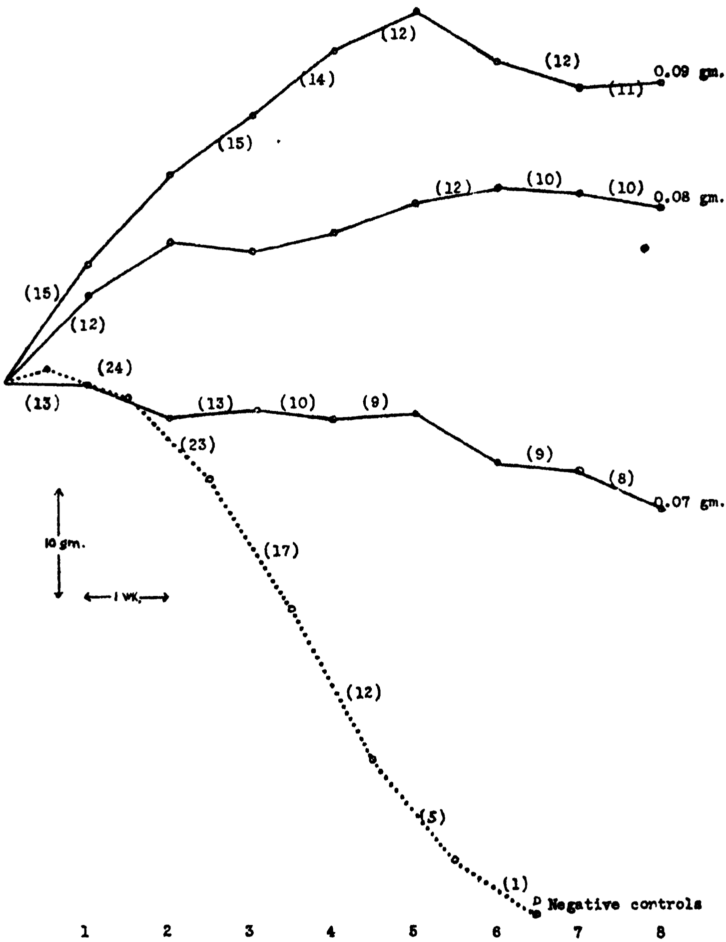

Chart I. Cumulative weight increases of rats fed medium sized leaves. The figures in parentheses represent the number of live animals averaged.

week are required to give an equal growth impulse when an equivalent quantity of the basal diet is consumed.

As shown in Chart III, 90 mg. of small leaves are more potent

than 100 mg. of large leaves and equal to from 120 to 150 mg. of large leaves.

Thus with average figures for gains in weights of rats over an 8 week period, it is demonstrated that weight for weight, small young New Zealand spinach leaves are more potent in vitamin A than are large, older leaves.

There are some discrepancies if one attempts to apply statistical methods in examination of the results. Variations in weight gains among individual animals in a group are very great and apparently the less vitamin provided the greater the variation. Perhaps this

TABLE I.

Vitamin A Tests on New Zealand Spinach Leaves.

Size of leaf.	New Zealand spinach per wk.	No of rats.	Weight at 28 days.	Initial weight.	Total period gain.	Food eaten per wk.	Rats surviving period.	Preliminary period.	Males.
sq. in.	*gm.*		*gm.*	*gm.*	*gm*	*gm.*	*per cent*	*days*	*per cent*
0 5	0 05	14	49	119	−15	54	50	38	43
	0 07	13	49	110	16	54	100	32	62
	0 09	14	49	105	41	72	93	34	64
4–5	0 07	15	49	119	−12	54	47	33	47
	0 08	12	50	109	16	53	83	29	58
	0 09	15	51	125	28	64	73	36	20
	0 12	4	52	103	45	74	100	26	50
8 10	0 10	7	46	124	5	60	29	37	71
	0 11	12	54	125	11	55	92	36	75
	0 12	7	47	142	37	69	100	45	29
	0 15	6	50	143	42	73	100	32	83

is to be expected in a method in which the animals are first reduced to a condition where the slightest changes in the diet or probably in the environment are made manifest by the physiological responses of the animals.

Again, if the curves for the animals fed 90 mg. of small and 90 mg. of medium sized leaves (Charts I and III) are compared, they are found to run parallel for the first 5 weeks, after which the rats on the small leaves continue to gain in weight and those on medium sized leaves lose weight. This might be due to the greater requirement of vitamin A of the second group of rats which on the

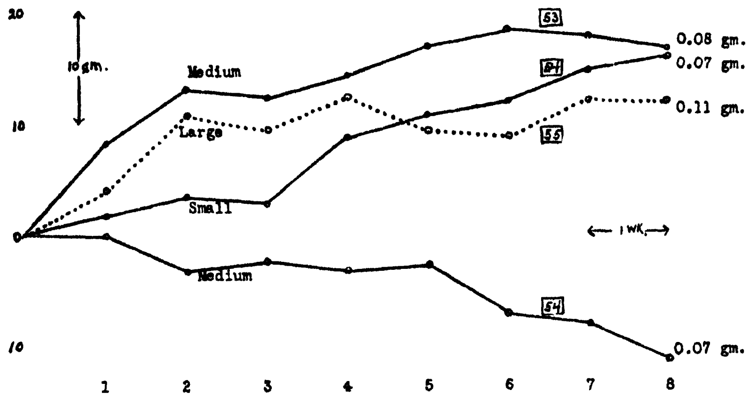

Chart II. Cumulative weight increases of rats on three sizes of leaves. The figures in squares represent the average consumption of basal diet in gm.

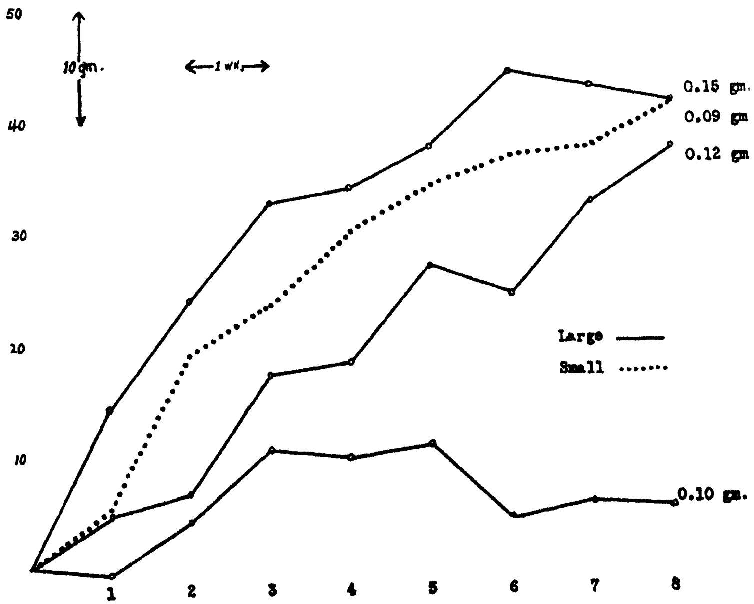

Chart III. Cumulative weight increases of rats on large leaves compared with one group on small leaves.

average are 20 gm. heavier. However, the difference between the two groups is also reflected in the food consumption figures, the second group losing its appetite for the basal diet earlier, as shown in Table II.

New Zealand spinach leaves (*Tetragonia expansa*) are thick when compared with such leaves as those of spinach (*Spinacia oleracea*). Especially is this true of the large succulent leaves that grow only on the short central axis of the plant; great variability was noticed in the thickness of these leaves and this property

TABLE II.

Weekly Consumption of Basal Diet of Rats Fed 0 09 Gm of New Zealand Spinach per Week

Wk	1	2	3	4	5	6	7	8
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm.*	*gm*
Small leaves fed	67 8	71 0	72 0	73 9	82 9	71 4	68 0	69 5
Medium " "	66 3	72 7	67 9	61 5	57 0	60 0	60 2	63 7

TABLE III

Relation of Leaf Weight to Upper Surface Area

Weight of leaf	Area of leaf	No of leaves	Ratio of weight to area	Relative thickness of leaves	Coefficient of correlation *
gm	*sq in*				
0 0612	0 23	48	26 52	1 00	0 992
1 588	4 71	43	33 76	1 27	0 985
3 525	8 62	29	40 88	1 54	0 790

* Weight considered as the independent variable and area the dependent variable

is brought out in the sixth column of Table III if comparison is made of large leaves with other leaves. Should vitamin A be associated with substances in the plant cells near the surface of the leaf, some of the variations in growth among the individual rats in a group might be due to differences in thickness in the vitamin-bearing food as well as to differences in the rats themselves. Effects upon growth are cumulative and in 8 week periods with many rats compensations for getting thicker leaves on some occasions are to be expected.

To determine the relation between weights of the green leaves

and their surface areas, many leaves of each size were picked, weighed quickly, and then measured. Average figures for the results are shown in Table III.

Thus on the average it would take 1.54 times as great a weight of large leaves as of small leaves for the same surface area, and 1.27 times the weight of medium leaves as of small leaves.

If the vitamin A content is accepted as equivalent in the quantities of leafy tissue fed rats whose growth curves are drawn in Chart II, the ratio of potency of small, medium, and large leaves is 1.57:1.14:1. If the quantities of New Zealand spinach leaves fed rats used for the two upper curves of Chart III are accepted as equivalent, the ratio of the potencies of small and large leaves is 1.67:1; if 0.09 gm. of small leaves were equivalent to 0.12 gm. of large leaves (see Chart III), the ratio would be 1.33:1.

This experiment then indicates that the vitamin A concentration of a New Zealand spinach leaf is directly dependent on its surface area.

SUMMARY.

The relative vitamin A content of New Zealand spinach leaves of three different sizes was determined by a rat growth method.

The potency of small leaves is greater than that of large leaves, if equal weights of the two supplement a vitamin A-free diet.

The weights and the corresponding surface areas of leaves of three sizes were compared and the ratio of the thicknesses of the three was calculated.

The ratio of the thicknesses is approximately the reverse of the ratio of the leaf potencies, indicating that the vitamin A content of leaves depends upon the surface area.

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THE RESULTS OF THE INGESTION OF CERTAIN CALCIUM SALTS AND OF LACTOSE.*

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The extensive use of calcium salts and of lactose for the relief of various symptoms ascribed to calcium deficiency has made a study of the effects of their administration advisable. The present report deals with the results of the ingestion of calcium chloride alone, of lactose alone, of calcium lactate alone and with lactose, and of calcium phosphate alone and with lactose on the blood picture and calcium-phosphorus balance.

The subjects were healthy male calves weighing from 104 to 145 kilos at the commencement of the experiments. The work was done in three experiments with three animals in each group. The animals were placed in the metabolism stalls and fed the basal ration for several days before the collection of excreta was begun. After a fore period of a week on the basal diet the supplement was added and fed for a week. This was followed by a 2nd week on the basal ration, after which the next supplement to be studied was included in the ration for a week. Exceptions were made to this routine in two cases; *viz.*, calcium chloride could be fed for only 4 days because of the disturbance it produced in the animals, and lactose in one case was fed for 2 weeks to insure the establishment of the effects of its ingestion. The basal ration was calculated to keep the animals just in equilibrium or a slight positive balance with respect to calcium and phosphorus.

Calcium and inorganic phosphorus in blood were determined by methods used in previous work (1).

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Feces and urine were collected separately in 24 hour periods. Each collection was weighed or measured, thoroughly mixed, and sampled for analysis.

200 cc. of urine and 50 cc. of HNO_3 were mixed to make a stock solution from which aliquots were taken. For the calcium, 50 or 100 cc. portions were evaporated on the steam bath and ashed at a low red heat in an electric muffle. The ash was taken up in dilute HCl, the silica dehydrated and removed, and the solution made up to 100 cc. The calcium was precipitated as the oxalate under the conditions recommended by Shohl (2) and titrated with permanganate. For the phosphorus, 10 cc. portions of the urine-HNO_3 solution were evaporated to dryness after the addition of a few cc. of $Mg(NO_3)_2$ solution and ashed at as low a temperature as possible. The ash was treated as before and the phosphorus determined colorimetrically by Briggs' modification of the Bell-Doisy method (3).

200 gm. portions of the moist feces were dried and weighed and the moisture content of the original material calculated. 5 gm. samples were ashed and the calcium and phosphorus in the solution determined by the same methods used for urine.

Serum Calcium and Inorganic Phosphate.

The effects of the various salts on the blood picture are shown in Table I. The effect of calcium chloride is not so pronounced as it should be because of the lack of samples during the period itself but those taken at the end of the period showed the highest values for calcium secured during the experiment. The phosphorus figures are inconclusive.

With lactose alone the calcium showed the unexpected tendency to fall and with two animals actually approached the low value of 8 mg. per cent. The phosphorus rose in a corresponding degree. 10 days after cessation of feeding the trend of both was towards the prefeeding level. Inouye (4) found that the administration of lactose raised the blood calcium and we have some unpublished results of the same character. Gross (5), on the other hand, failed to note such an increase. The present instance appears to be the first where a diminution occurs. Inouye's results were based on feeding periods of about a week and our own previous experiments were of about the same duration. In the present

TABLE I.

Blood Picture.

Experiment 1. Calf:						
Date.	S_1 Ca	S_1 P	S_2 Ca	S_2 P	S_3 Ca	S_3 P
July 9	10.6	7.40	11.0	7.36	10.3	6.87
" 11	10.6	8.02	10.5	6.80	11.2	7.10
" 14	10.2	5.85	10.7	5.50	10.8	5.55
" 17	12.4	8.56	15.7	6.25	12.3	6.38
" 20	11.0	8.93	11.1	8.74	11.4	8.45
" 23	11.7	8.86	11.7	7.66	11.7	8.56
" 26	10.0	9.47	11.0	7.76	11.0	8.28
" 29	11.6	8.39	11.3	7.57	12.1	8.01
Aug. 1	11.1	8.01	11.8	7.48	11.8	7.72
" 4	12.6	6.18	12.7	7.85	12.0	5.48
" 7	14.4	6.61	13.4	5.53	13.9	6.10
" 10	12.2	8.74	11.3	8.34	11.8	Lost.
" 13	12.4	9.06	12.0	9.33	11.8	8.92
" 14	11.3	8.33	11.8	7.06	11.4	6.75

Experiment 2. Calf:						
Date.	S_4 Ca	S_4 P	S_5 Ca	S_5 P	S_6 Ca	S_6 P
Nov. 8	Lost.	Lost.	10.8	4.91	10.7	4.16
" 9	9.2	8.08	Lost.	Lost.	11.8	6.25
" 16	13.3	5.00	12.7	4.30	Lost.	Lost.
" 17	11.2	5.58	11.0	4.25	10.6	4.47
" 19	12.5	5.21	12.4	5.68	12.2	5.44
" 21	11.7	Lost.	12.2	4.40	11.7	4.43
" 23	Lost.	5.72	Lost.	6.16	Lost.	5.36
" 25	8.9	4.81	10.1	5.00	10.4	4.69
" 28	9.7	4.73	10.2	4.94	11.1	4.80
" 30	9.9	4.78	11.4	4.88	11.4	5.92
Dec. 2	10.6	4.30	10.9	4.51	11.6	5.52
" 5	10.2	4.68	10.7	4.72	12.2	5.54
" 7	9.2	4.57	10.2	4.79	11.1	5.52
" 9	8.8	4.25	8.9	5.39	10.4	6.09
" 12	8.5	5.68	8.0	6.41	10.1	6.94
" 14	8.9	6.12	9.6	7.10	10.6	6.15
" 16	8.3	5.85	9.0	6.92	10.5	5.88
" 19	9.1	5.21	10.7	6.10	12.2	4.79

Experiment 3. Calf:						
Date.	S_7 Ca	S_7 P	S_8 Ca	S_8 P	S_9 Ca	S_9 P
Nov. 8	11.4	4.95	10.7	4.58	10.9	6.51
" 9	11.8	7.14	12.3	5.48	11.4	8.68
" 16	12.4	8.22	12.3	8.33	12.6	10.37
" 17	11.7	7.96	10.4	7.73	10.9	8.42
" 19	11.9	7.00	12.2	7.81	12.7	8.28
" 21	Lost.	Lost.	12.1	6.25	13.0	7.57
" 23	"	6.10	Lost.	6.72	Lost.	7.35
" 25	10.9	6.25	11.7	6.42	10.9	7.29
" 28	12.5	5.67	11.6	5.51	11.8	5.27
" 30	12.0	5.19	12.2	5.07	12.2	4.60
Dec. 2	12.3	5.59	12.4	4.98	12.6	5.07
" 5	11.7	7.68	11.5	8.06	Lost.	Lost.
" 7	12.4	6.79	11.5	7.01	11.2	8.02
" 9	11.6	5.68	11.6	7.31	10.4	7.57
" 12	11.2	5.68	11.4	6.58	11.7	7.35

Experiment 1.—From July 13 to end of experiment, 5 gm. of lactose per kilo were administered. From July 18 to 25, 0.7 gm. of bone meal per kilo was administered. From August 1 to 7, 1.5 gm. of calcium lactate per kilo were administered.

Experiment 2.—From November 12 to 15, 1.0 gm. of $CaCl_2$ per kilo were administered. From November 26 to December 10, 5.0 gm. of lactose per kilo were administered.

Experiment 3.—From November 12 to 19, 0.7 gm. of bone meal per kilo was administered. From November 26 to December 3, 1.5 gm. of calcium lactate per kilo were administered.

case the calcium values did actually show a tendency to rise somewhat for the first 10 days but the subsequent fall of calcium and rise of phosphorus figures are so distinct that one is tempted to look upon them as the real result of the carbohydrate feeding. There were no accompanying fluctuations in excreted calcium or phosphorus and no explanation offers itself.

Neither bone meal nor calcium lactate had any effect on the blood calcium. Though others have noted increased calcium values when lactate was fed, the magnitude of the results depended

TABLE II.

Per Cent of Calcium and Phosphorus of Supplement Excreted and Stored.

Excretion.	Basal.					Supplement.				
	Chloride.	Lactate.	Lactate and lactose.	Phosphate.	Phosphate and lactose.	Chloride.	Lactate.	Lactate and lactose.	Phosphate.	Phosphate and lactose.
Urine Ca	2.0	4.3	1.8	4.3	1.8	11.8	−0.1	1.3	0.9	0.1
" P	33.4	39.7	33.1	39.7	33.1	16.5*	−29.0*	−30.0*	45.8	21.5
Feces Ca	62.5	75.1	62.8	75.1	62.8	76.5	47.7	46.5	80.8	63.6
" P	45.5	48.4	44.4	48.4	44.4	15.4*	13.3*	0.5*	33.2	41.0
Total Ca	64.5	79.4	64.6	79.4	64.6	88.3	47.6	47.8	81.7	63.7
" P	78.9	88.1	77.5	88.1	77.5	31.9*	−15.7*	−29.5*	79.0	62 5
Stored Ca	35.5	20.6	35.4	20.6	35.4	11.7	52.4	52.3	18 3	36 3
" P	21.1	11.9	22.5	11.9	22.5	−31.9*	+15.7*	+29.5*	21.0	37.5
Stored and urinary Ca						23.5	52.3	53.6	19.2	36.4

* When the supplement contains no phosphorus the figures represent per cent of phosphorus in the feed.

on the prefeeding level and the amount fed. As the bloods of our animals were all showing calcium contents of about 12 mg. per cent, the absence of further increases is not surprizing. The phosphorus picture is, with bone meal, a characteristic one, showing the increase which usually accompanies the increased ingestion of this element. During the calcium lactate period there was a noteworthy fall in serum phosphorus, which is probably explainable by the increased fixation of phosphorus in the tissues. The same phenomenon was evident when lactose was fed with the calcium lactate and in this case there was a sharp rise in serum

calcium. This would agree with the theory that lactose augments calcium absorption though in the present instance the excretion figures afford no confirmation of this. There was no corresponding increase in serum calcium when lactose was fed with bone meal though there was an increased retention of the element.

Calcium and Phosphorus Metabolism.

The calcium and phosphorus in the excreta were determined and the results plotted. The feeding periods were of 7 days duration with the exception of that for calcium chloride but an inspection of the graphs showed that the effects of the supplements lasted about 3 days after the termination of the actual feeding period. Hence calculations are based on 10 day periods. The daily basal intake and excretion were figured from the fore period during which no supplement was fed and the metabolism due to the supplement was calculated by subtracting these basal figures from the total. The values obtained during the supplement feeding period are summarized in Table II and are calculated in per cent of intake. In the case of the non-phosphatic supplements the figures represent per cent of the phosphorus in the ration.

DISCUSSION.

Metabolism of Calcium Chloride, Calcium Lactate, and Calcium Phosphate.—Calcium chloride, calcium lactate, and calcium phosphate are effective in relieving tetany and in raising the serum calcium in the above order. Although at first sight their effectiveness would appear to be due to the relative ease with which they furnish calcium to the body and has frequently been so explained, (*cf.* Jansen (6)) the complicating factor of the production of an acidosis by calcium chloride has been recognized as of more or less significance in the response secured with this salt. Stewart and Haldane (7) attribute about half the increase in serum calcium caused by the ingestion of calcium chloride to the absorbed calcium and half to the acidosis. Gamble, Ross, and Tisdall (8) emphasize the possibility of much of the calcium in the chloride combining with phosphate and fatty acids of the food and so escaping absorption altogether. Our results appear to confirm these assertions.

The most noticeable feature about the results with calcium chloride is the great loss of phosphorus which the ingestion of this salt produces. Not only was all of the phosphorus of the basal ration excreted but the body reserves were drawn upon to a considerable extent. It has been shown that calcium chloride acts on the body in a manner similar to hydrochloric acid and an analogous loss of phosphorus has been shown to take place in the case of this latter substance (Fitz, Alsberg, and Henderson (9), Steenbock, Nelson, and Hart (10), Goto (11), Gamble and Ross (12), Scheer (13)). With hydrochloric acid most of the phosphorus is excreted in the urine. Goto claims that it is removed from the soft tissue and Gamble and Ross ascribe its escape to a depletion of body fluids analogous to that caused by the diuretic action of $CaCl_2$. In our experiments such a relationship is not evident since the loss of phosphorus and of water were not coincident. We have found in the literature no data on the excretion of phosphorus after ingestion of $CaCl_2$. Hence our results are of interest in affording information on this point. Apparently the action of calcium chloride does not resemble HCl in its effect on the route of the excretion of phosphorus, the increased elimination being equally distributed between the urine and feces. The explanation may be that the portion passing out by the gut has been combined with absorbed calcium and diverted to the bowel, whereas with hydrochloric acid in the absence of such a supply of calcium it passes out through the kidney. From the standpoint of the mechanism of its therapeutic action this is of some importance, but even on the assumption that all of the increase in fecal phosphorus accompanying calcium chloride ingestion is absorbed calcium, the amount of calcium absorbed from calcium chloride is only about 60 per cent of that absorbed from the lactate. Hence the estimate of Stewart and Haldane (7) that only half of the action of $CaCl_2$ is due to the absorbed calcium appears to be approximately correct. It may be that a part of the efficacy of calcium chloride in relieving tetany is due to this increase in phosphorus excretion. Phosphorus retention has been shown to be as constant a finding as lowered blood calcium in parathyroid tetany and the counteraction of this tendency may be as important as the raising of the blood calcium level.

The lactate presents a picture essentially different from that of

washed precipitate should be stirred up with alcohol, again centrifuged, the centrifuge tubes placed in a vacuum desiccator overnight, and the product weighed. The dry precipitate is then ground up with water for decomposition. Otherwise the original moist precipitate is suspended in 4 or 5 times its bulk of distilled water and decomposed with hydrogen sulfide. The sulfide must be well washed. The filtrate from the copper sulfide, which should be water-clear and completely colorless, is freed from H_2S by a stream of hydrogen, and then evaporated in a good vacuum at 40°. Evaporation should stop before the material is syrupy, the solution being removed from the vacuum flask when it measures, say, 6 to 8 cc., or thereabouts, for each kilo of yeast extracted. It is placed in a large crystallizing dish, mixed with half its volume of alcohol, a little more alcohol being poured upon the surface of the solution, and the dish then allowed to stand in a thoroughly evacuated desiccator containing fresh sulfuric acid. Crystallization may be somewhat slow to begin, but in from 24 to 36 hours foci of crystals will be seen and thereafter separation rapidly proceeds. If, as very rarely happens, no crystals have appeared by the time the solution has become syrupy, slight disturbance, such for instance as is involved in transference to a smaller crystallizing dish, is invariably followed by rapid crystallization. The solution of the peptide is sometimes apt to become supersaturated, but then if the familiar method of scratching the surface of the basin is adopted, it is always followed by a rapid separation of thick magma consisting of microscopic prisms.

In general it is found that the product thus obtained is so pure that if the solution be taken quite to dryness the residue gives good figures on analysis, or if successive crystalline fractions have been separated this remains true of the contents of the final mother liquors. More rarely a small syrupy uncrystallizable residue is obtained, from which however cuprous oxide precipitates a large proportion of the normal copper salt. Experience suggests that this is not necessarily because the original copper preparation was impure, but may be because in such cases the tripeptide has undergone slight decomposition owing, possibly, to the process of evaporation at 40° (*supra*) being, as the result of a poor vacuum, overprolonged.

The crystalline fractions should not require recrystallization

for purity. If however it be desired to recrystallize them, they must be dissolved in a minimum of water and, after the addition of alcohol, left in the desiccator as before. No solvent has been found from which more direct recrystallization is possible.

Owing to the nature of the substance, I have described its crystallization in (perhaps unnecessary) detail. As a matter of fact the product, although a tripeptide, crystallizes so readily that in dealing with it one need only remember that it is not very insoluble in the only medium at present available, namely, dilute alcohol.

However carefully the end-point is adjusted, precipitation by cuprous oxide as described above does not remove the whole of the tripeptide which is contained in the original mercury precipitate. It may happen indeed that no more than from 70 to 75 per cent is so precipitated. If the clear solution from which the first copper precipitate has been removed be again treated with the mercuric sulfate reagent (without the trouble to remove any excess of copper) and if the resultant precipitate be decomposed by H_2S and the solution as before be made 0.5 N with sulfuric acid, it yields on addition of cuprous oxide a further precipitate of the characteristic copper salt. This is not due solely to an increase in concentration, but largely to a readjustment of somewhat obscure equilibria in the solution. Precipitation may be yet incomplete however carefully the end-point is adjusted. A further (now small) quantity of the pure copper salt may be obtained after a second precipitation with mercury. On these lines upwards of 1 gm. of the crystalline product per kilo of yeast has been obtained.

At the risk of some repetition I feel it may be helpful to describe a separation, as actually carried out, in a case where a record was kept of the stages involved.

12 kilos of French pressed yeast were boiled up in a large enamelled iron basin with 12 liters of water containing 0.1 per cent of acetic acid. The extract was kept boiling for 3 or 4 minutes and the thick brew then transferred while still hot to six large Buchner funnels in which the filter papers were covered with a thin layer of kieselguhr. A moderate vacuum being maintained in the filter flasks, the residue became completely dry overnight. It was again boiled up with 5 liters of acidulated water and returned to the filters. The final residue was sucked dry but not further washed.

To the mixed filtrates (16.5 liters) 250 cc. of saturated neutral lead acetate solution were added, and then, more gradually and with constant stirring, 2400 cc. of the mercuric sulfate reagent. When the mixture had stood overnight the mercury precipitate settled well, the supernatant fluid, though not quite clear, containing only a negligible quantity of suspended material. The fluid was syphoned off and the precipitate transferred to a Buchner funnel. It filtered well with the pump, though somewhat slowly. It was washed thrice with distilled water and then transferred to a mortar and rubbed into a thin uniform paste. This was transferred to a 5 liter flask and the suspension of the precipitate finally made to measure about 2 liters. The flask being provided with cork and tubes, the decomposition by H_2S delivered from a Kipp was carried out with the exit tube closed, the flask being frequently shaken. After about 40 hours decomposition was complete, as evidenced by the absence of bubbles in a wash bottle when the flask was vigorously shaken. The mercury precipitate was filtered off and washed thrice at the pump. H_2S was removed from the filtrate, the greater part by aeration, and the last traces by a stream of hydrogen. The solution now measured 2300 cc. and was acidified by the addition of 25 cc. of strong sulfuric acid (diluted before addition). The flask was heated on the water bath, the fluid being kept in motion till its temperature reached 45°. 3 gm. of Cu_2O in fine aqueous suspension were gradually added, the flask being well shaken between each addition. The precipitate showed a silky sheen and soon became bulky. The contents of the flask were transferred to a tall beaker and in the course of a few hours the precipitate had completely settled, when the clear fluid was syphoned off. The precipitate and associated fluid were then centrifuged thoroughly and the fluid returned to the main bulk of solution. To the latter 0.1 gm. of Cu_2O was added and a small, but appreciable, further quantity of precipitate formed with the usual crystalline appearance. On standing however instead of settling, this small fraction became completely redissolved.

The first copper precipitate was washed at the centrifuge with oxygen-free distilled water until free from any trace of sulfuric acid. Contained in four tubes, each holding 45 cc. of wash water, it had to be centrifuged ten times before the acid was wholly

removed. It was finally stirred up with 97 per cent alcohol, again centrifuged till as dry as possible, and then, still in the centrifuge tubes, transferred to a vacuum desiccator. When dry the copper salt, still completely colorless, weighed 7.9 gm.

To the solution from which this first fraction of the copper salt had been removed mercuric sulfate was added and the resultant precipitate washed and decomposed. To the filtrate, again made about 0.5 N with H_2SO_4, 0.4 gm. of cuprous oxide was added. The typical silky precipitate when separated, washed, and dried weighed 1.6 gm. On similar lines another 0.48 gm. was obtained. The total yield of practically 10 gm. of the copper compound (= 0.69 gm. of tripeptide per kilo of moist yeast) was in this case below the average. It should be understood however that the substance is strongly adsorbed on the successive sulfide precipitates. Complete washing takes much time and when a supply of materials is alone required may not in the end prove economical. A more nearly accurate estimate of the amount of tripeptide actually present is got by using small quantities of yeast. Thus from 2 kilos of fully extracted yeast, with thorough washing of successive precipitates, 2.1 to 2.4 gm. of tripeptide have been obtained.

Separation from Blood Corpuscles.—Before the cuprous compound method had been discovered a preparation made from ox blood by the modification of the original method had been found to yield the characteristic diketopiperazine of glycine and cystine on being boiled with water, and from the products of its acid hydrolysis glutamic acid, glycine, and cystine were isolated. Later the new copper method was applied to a remainder of this preparation and the presence of the tripeptide demonstrated. The new technique was then applied to blood itself and the tripeptide easily isolated pure. Only one such preparation has so far been made however, and as the optimal conditions for the isolation from blood have not been fully studied, the yield obtained must not be taken as evidence for the amount of the tripeptide actually contained in the corpuscles.

20 liters of whole ox blood were mixed with 10 liters of water and then with 7 liters of 0.1 N sulfuric acid and the mixture heated to 80°. The coagulum was filtered off and to the cooled filtrate the mercuric sulfate reagent was added. Whether or not because of a difference in the buffers present the blood preparation at this

stage does not yield the mercury precipitate so readily as does a yeast extract. Sodium hydroxide must be added till the acidity of the solution is considerably reduced. By adding the mercuric sulfate gradually, alternating the additions with small additions of alkali, the point of maximal precipitation can be determined without much difficulty. When, in the present case, this point was attained, the precipitate was filtered off, washed, and decomposed. The solution was for a second time precipitated with mercury and the precipitate treated as before. To the solution thus finally obtained (900 cc.), when freed from H_2S and warmed to 45°, cuprous oxide was cautiously added. The characteristic silky precipitate of the copper compound quickly formed. It was allowed to settle and then centrifuged off and thoroughly washed on the centrifuge with oxygen-free water. Dehydrated with alcohol and dried in the vacuum desiccator, it weighed 0.92 gm. After the copper salt was decomposed the colorless filtrate from the copper sulfide was evaporated *in vacuo* and, finally, in the desiccator. It readily yielded crops of crystals and the product so obtained agreed in every respect with the product from yeast. It yielded consistently accurate analytic figures for the tripeptide, as will be seen below.

Note on a Modification of the Original (1921) Method of Separation.

The following brief account of this method is given because certain experiments described in this paper were carried out on products obtained by its use, but also because these less pure products, though they certainly contained a large proportion of the tripeptide, show differences of behavior which may have significance (p. 282).

The yeast or tissue extract was first precipitated by the acid mercuric sulfate solution. The mercury was removed as sulfide and sulfuric acid removed from the filtrate, the solution being next precipitated by neutral lead acetate with careful avoidance of excess. The lead precipitate was ground up with 0.5 N sulfuric acid and successively extracted with this until the extracts no longer gave a nitroprusside reaction. The filtrate from the lead sulfate was precipitated by phosphotungstic acid added in quantity somewhat larger than that required to produce a maximal precipitate. The filtrate was cooled with ice and the excess of phosphotungstic acid removed with ice-cold barium hydroxide solution. The filtrate from the barium phosphotungstate was precipitated by mercuric sulfate; this precipitate was decomposed, and another lead precipitate obtained. The latter, decomposed as usual, yielded the solution of the final product. This was concentrated *in vacuo* at 40° and the product (with or without previous oxidation to the disulfide form) thrown out of solution with alcohol.

2. Analytical Data.[7]

Metallic Content of the Pure Copper Compound.

The following data were from preparations which had been washed at the centrifuge with oxygen-free water, then with alcohol, and, after standing in a well evacuated desiccator, dried to constant weight *in vacuo* at 50°. Only with such precautions is oxidation of the cuprous copper completely avoided. The dried products should show no blue color. All the figures given were obtained by igniting the compound in a stream of oxygen and weighing the CuO.

Product	A.	4.931 mg.:	1.102 mg. CuO.	Cu =	17.45 per cent.
"	B.	6.130 " :	1.290 " "	"	16.80 " "
"	C.	5.341 " :	1.181 " "	"	17.41 " "
"	D.	8.110 " :	1.746 " "	"	17.20 " "
"	E.	100.00 " :	21.540 " "	"	17.21 " "
Calculated for $C_{10}H_{16}N_3O_6Cu$					17.26 " "

Crystalline Tripeptide.

Preparation A (Yeast).—In this 5 gm. of the copper were decomposed and two successive fractions of the crystalline product were analyzed. The mother liquor from these was taken right to dryness and the crystalline residue analyzed. The products were first dried in a vacuum desiccator, and then, till of constant weight, at 50° in an evacuated tube connected with a bulb containing P_2O_5.

Fraction 1 (2.2 Gm.).

	C	H	N	S
4.773 mg.: 6.840 mg. CO_2 and 2.30 mg. H_2O	39.11	5.45		
3.180 mg.: 0.366 cc. N at 21° and 761 mm			13.38	
5.570 mg.: 4.260 mg. $BaSO_4$				10.50

[7] The nitrogen and sulfur determinations were made by A. Colwell of this laboratory; the carbon and hydrogen by Schoeller of Berlin-Schmargendorf.

Fraction 2 (1.19 Gm.).

	C	H	N	S
4.951 mg.: 7.010 mg. CO_2 and 2.42 mg. H_2O	38.80	5.45		
3.288 mg.: 0.380 cc. N at 20° and 761 mm			13.43	
5.715 mg.: 4.435 mg. $BaSO_4$				10.65

Residues from Mother Liquor.

	C	H	N	S
5.181 mg.: 7.34 mg. CO_2 and 2.62 mg. H_2O	38.64	5 61		
6.477 mg.: 4.957 mg. $BaSO_4$				10.51
Micro-Kjeldahl			13 41	

The following preparations from yeast were made by colleagues.

Preparation M.

	C	H	N	S
4.303 mg. required 4.11 cc. 0.01 N acid			13 37	
5.602 mg.: 4.249 mg. $BaSO_4$				10.42

Preparation P.

	C	H	N	S
4.485 mg. required 4.39 cc. 0.01 N acid			13 40	
5.556 mg.: 4.237 mg. $BaSO_4$				10.47

Preparation C (from Yeast of Different Provenance).

	C	H	N	S
Fraction 1	38 91	5 41	13 56	10 41
" 2			13 41	10 45

Preparation from Blood.

	C	H	N	S
4.610 mg.: 6.590 mg. CO_2 and 2.38 mg. H_2O	39.00	5.71		
6.286 mg.: 4.882 mg. $BaSO_4$				10.67
4.427 " required 4.24 cc. 0.01 N acid			13 41	
Mean of all analyses	38 89	5.53	13 42	10.51
Calculated for $C_{10}H_{17}N_3SO_6$	39.09	5 54	13 68	10 42

3. Results of Acid Hydrolysis.

Before the crystalline product had been prepared a somewhat long apprenticeship had been served in isolating the amino acids derived from the earlier less pure preparations. Only cysteine (or cystine), glycine, and glutamic acid were found, and a satisfactory technique for isolating these, with at least approximately quantitative results, became familiar. In the case of the particular amino acids in question preliminary esterification served no good purpose from a quantitative point of view; and for the method used (removal of cysteine by precipitation with mercury, and reliance upon fractional crystallization for the separation of the others) the employment of relatively small amounts came to be preferred.

The pure tripeptide has been hydrolyzed both with hydrochloric and with sulfuric acids. The former has the disadvantage that in removing the acid remaining after evaporation with silver sulfate or silver oxide it is impossible to avoid simultaneous precipitation of part of the cysteine; the use of sulfuric acid of course needs care in the avoidance of loss when one is dealing with the large barium sulfate precipitate.

In respect to the isolation of glutamic acid and glycine the results of hydrochloric acid and sulfuric acid hydrolysis have been so similar that the latter alone need be described in detail.

2 gm. of a crystalline product were boiled for 16 hours with 30 cc. of 25 per cent sulfuric acid. The greater part of the acid was removed with baryta and the barium sulfate precipitate very thoroughly washed. The solution was then precipitated with the acid $HgSO_4$ reagent and practically every trace of cysteine thus removed. From the filtrate the mercury was removed as sulfide and the sulfuric acid (exactly) as barium sulfate. The solution was then evaporated and the successive crystalline fractions which separated were filtered off quantitatively through very small filters. The first three fractions, weighing together 0.726 gm., proved to be glutamic acid analytically pure. Late fractions weighing 0.250 gm. consisted of pure glycine. Intermediate fractions however with an average nitrogen content of 17.5 per cent (glycine 18.66, glutamic acid 9.52) were resistant to further separation. Each yielded however, when treated with picric acid, a

copious precipitate of glycine picrate. In making the yield of this approximately quantitative advantage was taken of the detailed study of Levene and Van Slyke (7). The combined fractions were dissolved in distilled water (1.5 cc. for each decigram) and picric acid added in the proportion of 1.8 parts to 1 part of the material. When the mixture was boiled the picric acid went completely into solution. When cooled to room temperature, the solution was sown with a crystal of pure glycine picrate and immediately became nearly solid with a crystalline precipitate. After standing on ice for an hour, the mother liquor was drained off, the crystals washed with a little 50 per cent alcohol, and then with absolute alcohol and ether. Without further treatment the picrate so obtained was completely pure. When dried it melted sharply at 202°, after softening at 200°. The melting point was not altered by admixture with pure glycine picrate. The amino N was determined in a Van Slyke apparatus. When multipled by the factor 0.94 the result obtained was 7.51 per cent. Calculated for $(C_2H_5NO_2)_2C_6H_3N_3O_7$, 7.39 per cent.

On concentration of the filtrate another small yield of glycine picrate was obtained and finally, after acidification and removal of the picric acid with ether, a crystalline fraction consisting of glutamic acid.

The non-crystalline mother liquors from the crystalline fractions as first obtained (see above) gave a residue weighing 0.14 gm. This largely consisted of glycine, as shown by the yield of pure picrate.

The mercury precipitate obtained as above was decomposed by H_2S and the sulfuric acid removed with baryta. The solution when quite free from H_2S was made alkaline with ammonia, a trace of iron being added to secure oxidation of the cysteine to cystine, then evaporated slowly on the water bath, and allowed to stand. The cystine which was finally separated weighed 0.710 gm. The uncrystallizable mother liquor from this gave a residue weighing 70 mg. only.

By the procedure described 0.710 gm. of cystine (90.0 per cent of theory for tripeptide) was obtained analytically pure; of pure glutamic acid 0.810 gm. (84.5 per cent of theory); and of glycine, either weighed pure or calculated from the picrate 0.3900 gm. (92 per cent of theory). The small deficiencies were due to manipula-

tive losses. If the data be scrutinized, it will be seen that no other amino acid could have been present.

Analyses.

		N	S	C
Cystine fraction.	Found....	11.48	26.20	
	Calculated.........	11.57	26.44	
Glutamic fractions (combined).	Found..............	9.44	6.20	40.71
	Calculated.........	9.52	6.12	40 82
Glycine fraction.	Found..............	18.54		
	Calculated.........	18.66		

The evidence for the purity of the glycine picrate is given above.

IV. Determination of Amino and Carboxyl Groups, and of the Minimal Molecular Weight, by the Acidimetric Titration Technique.

By Leslie J. Harris.

An extension of the theory of titrations was elaborated some years ago (Harris, 1923, etc.[1-5]) as a result of which it became possible to determine acids or bases which had previously been considered too weak to fall within the scope of acidimetric methods. It was shown, in particular, that the amino as well as the carboxyl groups in amino acids, polypeptides, and the like, could be determined by acidimetric titrations, and several alternative procedures for this purpose were described. The applications of the new technique were also shown to include methods for (1) determining combining weights, (2) estimating certain individual amino acids (or homologous series of amino acids) when present in complex mixtures of amino acids, (3) determining polypeptides or proteins volumetrically, and (4) measuring dissociation con-

[1] Harris, L. J., *Proc. Roy. Soc. London, Series B*, **95**, 440 (1923).

[2] Harris, L. J., *Proc. Roy. Soc. London, Series B*, **95**, 500 (1924).

[3] Harris, L. J., *J. Chem. Soc.*, **123**, 3294 (1923).

[4] Harris, L. J., *Biochem. J.*, **17**, 693 (1923); *Nature*, **115**, 119 (1925); *J. Soc. Chem. Ind.*, **44**, *Chem. and Ind. Rev.*, **3**, 1016 (1925); *Proc. Roy. Soc. London, Series B*, **97**, 364 (1925).

[5] Harris, L. J., *Proc. Roy. Soc. London, Series B*, **104**, 412 (1929).

stants. As of further direct interest in connection with the present note, it should be added that among other deductions it was shown also, for the first time, that the protein molecule is constituted as a "zwitterion,"[6] and that the peptide linkages, contrary to former supposition, are incapable of combining with acids or alkalies under any of the conditions examined.

As far as the general principle of these methods is concerned, it is sufficient here to say that any single amino acid (or any mixture of ampholytes) is regarded as equivalent to a mixture of single simple mono-acids and bases having the same separate dissociation constants as the several titration dissociation constants of the body in question, and therefore that when one is dealing with weak acids or bases, or when one is titrating to a sufficiently highly acid or alkaline end-point, the "strength" of the *water*, present as solvent, is comparable with the "strength" of these other weak acids or bases, and hence cannot be neglected; *i.e.*, the *water* present must be regarded as one of the *constituents* of the mixture.

Below is described the application of these methods to glutathione. Since the Van Slyke gasometric method[7] for amino groups gives irregular results with many peptides, often also with sulfur compounds, no less than with glycine derivatives, it would appear that the titration methods offer the only alternative at present available for gaining that knowledge of the number of amino and carboxyl groups, prenecessitated in arriving at the structure of the body.

The several methods described below all comprise simple titrations to some sharp color change with a pH indicator. (When applied to a large number of amino acids—and also to a few peptides—these same titration methods have invariably been found to give satisfactory results, generally to within 1 or 2 per cent of the theoretical.[8]) To obtain fullest information, such colorimetric estimations were studied, it will be remembered, in conjunction with complete titration curves, in water, formol, etc. The latter aspect in the present tests has been undertaken by Pirie and Pinhey and is described by them separately.[9]

[6] Similarly, isoelectric oxidized glutathione (G_2S_2) appears to be a complex zwitterion, with four ionized groups.

[7] Van Slyke, D. D., *J. Biol. Chem.*, **9**, 185 (1911).

[8] Except that arginine sometimes behaves as a mono-base rather than a di-base mono-acid.

[9] Pirie, N. W., and Pinhey, K. G., *J. Biol. Chem.*, **84**, 321 (1929).

All the findings to be recorded were obtained upon crystalline specimens of (reduced) glutathione which had been isolated by the newer method described by Hopkins in the present paper.

1. Estimation of NH_2 and COOH by the Alcohol Titration Method, (A) NH_2.

This method while having a slightly lower percentage accuracy than other methods to be described below, is marked by extreme simplicity and rapidity. Martens,[10] who has recently submitted it to a critical study, using not only amino acids but also peptides, concludes: "La méthode procure des résultats très précis pour tous les acides aminés. . . . frappé par la précision des résultats, séduit la simplicité du mode opératoire. . . . Elle a sur la méthode de Sørensen [et de Foreman] des avantages marqués."

Method.—A suitable weight of the glutathione (about 0.5 or 0.1 mg. equivalent) is taken and dissolved in a small quantity of water (*e.g.*, enough to make a 0.1 M solution); next sufficient alcohol may be added to make a final concentration of about 80 per cent; and, then from a micro burette that amount of soda (best, N) is run in which is required to titrate all the carboxyl groups present, as determined by a separate duplicate titration (Section 2, below). Finally methyl red indicator is added, and the amino groups are then determined by titrating with HCl (best, N) to a sharp change from yellow to orange (to match aqueous pH virage of about 5.4). (Formaldehyde must be absent in this titration.) (The titrations with N acid or alkali are conveniently carried out in a new type of micro burette which is graduated in 0.01 cc. intervals, holds a total of 2 or 5 cc., and is conveniently filled from the top like an ordinary macro burette.)

Note (a). On an Alternative Method.—When one is dealing only with known amino acids, *and in the absence of tyrosine,* it was shown in the early paper[2] that the above procedure could for convenience be somewhat shortened by performing the two titrations upon the same specimen. The carboxyl titration was first made with NaOH against thymolphthalein, and then in continuation the amino determination was made by a back titration with HCl against methyl red. In the presence of the weakly basic phenolic OH of tyrosine, however, the preliminary

[10] Martens, R., *Bull. Soc. chim. biol.*, **9**, 454 (1927).

titration with NaOH naturally gives a high reading (since part of the OH titrates) so that the back titration with HCl was found to be increased by the same amount. The shortened procedure is, then, clearly *less* advisable when one is investigating the behavior of unknowns, more definitely as in the present instance when the weakly basic —SH of glutathione is closely comparable in weak basicity with the phenolic OH of tyrosine.[11] The preliminary titration of carboxyl groups with soda should therefore be made by the alternative method described in my early paper; *i.e.*, (1) to phenolphthalein in 80 per cent alcohol and with formalin, instead of to thymolphthalein and without formalin; and (2) on a duplicate, since the subsequent amino titration is best performed in absence of formalin.

Note (b). On the Possibility of Omitting Alcohol in the Back Titration.—A small blank correction is made for the amount of soda required to take the solvents and indicator alone over the same pH range. This blank correction is naturally the smaller (and the titration correspondingly the more accurate) when the HCl titration is carried out in the absence of alcohol instead of with an excess of alcohol. Such an omission is generally permissible, indeed sometimes slightly advantageous in intensifying the sharpness of the end-point, when the NaOH titration is performed on a separate duplicate in advance, according to the slightly lengthened technique here adopted. The NaOH and HCl titrations give rise to the following type reactions, respectively, when stated in the zwitterion mode

y amino acid, containing:
x amino groups.
y carboxyl groups.

$$\xrightarrow{y\ \mathrm{NaOH}} \mathrm{R}\begin{cases}(\mathrm{NH_2})_x\\(\mathrm{COO^-})_y\end{cases} + y\ \mathrm{Na^+} \xrightarrow{x\ \mathrm{HCl}} \mathrm{R}\begin{cases}(\mathrm{NH_3^+})_x\\(\mathrm{COO^-})_y\end{cases}$$

Ca. pH > 12, in aqueous solution (neutral to phenolphthalein or thymolphthalein in alcohol).

Ca. pH 5.4 (neutral to methyl red).

A detailed setting out of the different possible types of amino acid—acidic, basic, and neutral—will be found in the earlier paper,

[11] For glutathione pK_{SH} = 9 8; for tyrosine pK_{OH} = 9 7 or greater.

where the alternative classical mode of representation is employed in place of the present zwitterion style. The method may be said to depend on the fact that the following bodies in solution have a reaction near pH 5.4 (pK of methyl red): the monoamino-monocarboxylic acids, the monosodium salts of the dicarboxylic monamino acids, the monohydrochlorides of the diaminomono-amino acids.[12] The results of the analyses are given in Table A.

Results.—It will be seen that the titration values *found* are in very close agreement with those *calculated* (*i.e.*, for a tripeptide consisting of cysteine, glycine, and glutamic acid). With the

[12] The criticism might possibly be raised that there may exist, for all we know, among ampholytes as yet unisolated, some having pK values so widely divergent from the normal pK range of all the known ampholytes as to give inaccurate titration readings in this method. It is therefore worth recording that the technique in question has already been applied experimentally, so far with remarkably accurate results, to practically all the known natural amino acids, as well as to a number of polypeptides. Thus, giving our fullest available list of forty-three instances here appended, we see that the calculated error is always so small as to lie well under 1 per cent in all except the five instances where the slightly higher figures are cited. (We are here referring to titrations to pH 5.4 *without alcohol*,—the similar, or in some cases even better, experimental accuracy obtained *in the presence of alcohol* has already been recorded for the first seventeen of these bodies.[3,10].

Glycine, alanine, valine, leucine, phenylalanine, tryptophane, cystine, cysteine, proline.

Aspartic acid (error = + 2 per cent, but much less in alcohol), glutamic acid (error = + 4 per cent, but much less in alcohol), tyrosine (see text).

Lysine, arginine (see foot-note above), histidine (100 per cent of amino plus 90 per cent of imino in aqueous solution, but 100 per cent of amino only in alcohol).

Glycyl-glycine, leucyl-glycine.

Serine, isoleucine, norleucine, oxyproline, β-hydroxyglutamic acid (error = + 6 per cent in aqueous solution, ? less in alcohol), α-, γ-, and δ-amino-*n*-valeric acids, sarcosine.

Alanyl-alanine, alanyl-glycine, aminobutyryl-α-aminobutyric acid, glycyl-alanine, glycyl-asparagine, glycyl-leucine, glycyl-valine, glycyl-sarcosine, leucyl-asparagine, leucyl-isoserine, sarcosyl-sarcosine.

Aspartyl-glycine (error = + 12 per cent in aqueous solution, ? less in alcohol), glycyl-aspartic acid (error = + 10 per cent in aqueous solution, ? less in alcohol).

Glycyl-glycyl-glycine, alanyl-glycyl-glycine.

Glycyl-alanyl-alanyl-glycine.

Leucyl-octaglycyl-glycine.

	Specimen 1, F.G.H.*	Specimen 2, F.G.H.	Duplicate of Specimen 2.	Specimen 3, N.U.M.
	gm.	*gm.*	*gm.*	*gm.*
Weight of glutathione taken	0.0614	0.0500	0.0500	0.0601
	cc.	*cc.*	*cc.*	*cc.*
Water added (CO_2-free)	1	0	0	1
EtOH added	20	0	20	8
Standard NaOH run in (*i.e.* theoretical amount as determined in Section 2)	4.1† × 0.1 N (Macro burette.)	3.258‡ × 0.1 N (Micro burette.)	3.258‡ × 0.1 N (Micro burette.)	0.397† × N (Micro burette.)
Phenolphthalein (0.5 per cent in 50 per cent alcohol)	1	0	0	1
Methyl red (0.02 per cent in 60 per cent alcohol)	1	0.1	0.5	1
HCl blank	0.15	0		0.005
Found.				
Standard HCl required (corrected for blank)	**1.95, 2.00** × 0.1 N (Macro burette.)	**1.605** × 0.1 N (Micro burette.)	**1.605** × 0.1 N (Micro burette.)	**0.205** × N (Micro burette.)
Sensitiveness of end-point color change, in cc. HCl added	<0.05	<0.02	<0.03	<0.01
Calculated.				
For tripeptide of cysteine, glycine, glutamic acid	**2.00** × 0.1 N	**1.629** × 0.1 N	**1.629** × 0.1 N	**0.196** × N
	per cent	*per cent*	*per cent*	*per cent*
Maximum divergence. Between "found" and "calculated" readings	**<−2.5**	**−1.5**	**<−1.8**	**+4.6**

* Specimens 1 and 2 were both prepared by F. G. H.; Specimen 3 by N. M. Meldrum. Hopkins' method was used for all three specimens.

† NaOH blank was included here, and hence increased by the same amount as the HCl blank (*i.e.* in the back titration).

‡ NaOH blank was omitted.

most sensitive conditions, the divergence falls within 1.5 per cent of the reading.

2. Estimation of NH_2 and COOH by the Alcohol Titration Method, (B) COOH.

Although according to Willstätter and Waldschmidt-Leitz[13] the COOH of polypeptides may be titrated quantitatively in 40 per cent alcohol (against phenolphthalein), nevertheless preliminary trials with glutathione showed that the method gave extremely *low* readings. Even when the alcohol was increased up to 95.5 per cent,[14] 86 per cent only of the theoretical was reached (titrating to the first visible color change); or only 93.5 per cent of the theoretical, when titrating in 97 per cent alcohol (and to a fuller pink). On the other hand titration to thymolphthalein in alcohol gave, as anticipated, *high* results, owing to the ionization of the weakly basic - SH group. Accordingly the method previously described by the writer, which is a convenient, very slight modification of Foreman's COOH titration method, was used. It has previously given accurate results when applied to all the better known amino acids and to several peptides.

Method.—A 0.1 mg. equivalent of glutathione, more or less, is dissolved in water to make say 1 cc. of 0.1 M solution, to which are added about 8 cc. of alcohol, 1 cc. of formalin, and 1 cc. of phenolphthalein indicator. The mixture is then titrated to a sharp end-point (change from colorless to red) with 0.1 N, or preferably N, NaOH, with use of the micro burette already alluded to. The final alcohol concentration should be about 80 per cent. A small blank correction is made for the solvents.

Analytical Details.

1. First specimen (F. G. H.); macro titration, 0.1 N NaOH.
   0.0614 gm. glutathione (F. G. H.)
   1.0 cc. water (CO_2-free)
   20.0 " alcohol (97 per cent)

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[13] Willstätter, R., and Waldschmidt-Leitz, E., *Ber. chem. Ges.*, **54 B**, 2988 (1921).

[14] 97 per cent alcohol is required in the Willstätter method when free amino acids rather than peptides are to be titrated.

1.0 cc. phenolphthalein (0.5 per cent in 50 per cent alcohol)
Required 3.8 cc. 0.1 N NaOH
Added 1.0 cc. of formalin just neutralized to phenolphthalein
Required 4 1 cc. 0 1 N NaOH
Less 0 1 " blank correction =
4.0 " (corrected)
2. Duplicate. 4.0 " "
Theoretical for tripeptide, cysteine, glycine, glutamic acid... **4.0** cc.
Found.. **4.0** "
End-point is sensitive to <0.1 cc., hence "found" = **100** per cent ± <**2.5** per cent of "theoretical."

3. Second specimen (N. U. M.); micro titration, N NaOH.
0.0578 gm. glutathione (N. U. M.)
1 0 cc. water (CO_2-free)
8.0 " alcohol (absolute)
1 0 " phenolphthalein (0.5 per cent in 50 per cent alcohol)
Titrated from micro burette
Required 0.35 cc. N NaOH
Added 1.0 cc. of formalin just neutralized to phenolphthalein
Required 0.38 cc. N NaOH
Less 0.005 " blank correction
0 375 " (corrected)
Theoretical for tripeptide, cysteine, glycine, glutamic acid. **0.375** cc.
Found.. **0.375** "
End-point is sensitive to <0 01 cc., hence "found" = **100** per cent ± <**2.7** per cent of "theoretical."

Results.—The readings agree with the theoretical values calculated for the tripeptide within the experimental error of the method (*viz.* about 2.5 per cent).

3. Estimation of the Second COOH, by Titration in Water.

It was shown [1,3] that the second (*i.e.*, unbalanced) carboxyl group in dicarboxylicmonoamino acids, or the second (*i.e.*, unbalanced) amino group in diaminomonocarboxylic acids, can be estimated with great accuracy by titrating to a sharp color change to a pH slightly more acid than 7. This principle was made use of to estimate both the diamino and the dicarboxylic acids when present simultaneously in mixtures with other amino acids. It provides, in general, a method of determining unbalanced carboxyl or amino groups (*i.e.*, carboxyl in excess over amino, or *vice versa*).

It will now be understood that a titration to phenolphthalein

(pH 8.3), which is so commonly used for estimating single organic mono-acids, is liable to give erroneous results when applied to di-acid mono-bases or di-base mono-acids, or polyampholytes in general, since it estimates a fraction of a second group as well. Thus with glutathione, titration to a faint pink with phenolphthalein (? about pH 8.2) was found to give 115 per cent of the theory, or, to a more distinct color, a still higher figure.

Analytical Details.

First specimen (F. G. H.).
0.0614 gm. glutathione (F. G. H.)
1 0 cc. water (CO_2-free)
0 5 " brom-thymol blue indicator
Titrated from micro burette

Required	0.205	cc. N NaOH
Theoretical (for the tripeptide)	0.200	" " "
Method sensitive to	0.005	" " "

Another preparation (N. U. M.).
0 0304 gm. glutathione (N. U. M.)
1.0 cc. water (CO_2-free)
0 5 " brom-thymol blue
Titrated from micro burette

Required	0.10	cc. N NaOH
Theoretical (for the tripeptide)	0.10	" " "
Method sensitive to	0.005	" " "

Results.—These agree with theoretical (tripeptide) within the experimental error of the method (0.25 per cent) and are in confirmation of conclusions reached above in Sections 1 and 2.

4. Estimation of Total COOH, "Neutral COOH," and Hence NH_2 by Means of Formaldehyde Titrations.

As has been pointed out by Brown,[15] and by the present writer, the method of performing a formol titration (*cf.* Schiff, 1900;[16] Sörensen 1908[17]) as described in certain text-books gives fallacious results. One is directed, according to these, first to adjust the unknown solution to phenolphthalein (Stage 1), next to add neutralized formalin, and then to retitrate to phenolphthalein (Stage 2). The error arises in the first adjustment of

[15] Brown, J. H., *J. Bact.*, **8**, 245 (1923).
[16] Schiff, H., *Ann. Chem.*, **319**, 59 (1901).
[17] Sörensen, S. P. L., *Biochem. Z.*, **7**, 45 (1908).

the unknown (Stage 1) since quite an appreciable fraction of the carboxyl to be estimated becomes neutralized during this stage, so that the titer found after addition of formalin (Stage 2) is short by the same amount.[18] From a recent detailed study[5] it is clear that an accurate estimation of total carboxyl can be obtained if one adds the specimen, not previously neutralized, to already neutralized formalin and titrates direct with NaOH. (If sufficient formalin be added, it is no longer necessary to titrate to so alkaline an end-point as phenolphthalein, and indeed results of somewhat greater precision are then possible, the relative blank corrections being less.)

Titration to pH 6 *ca.*, before addition of formalin gives the excess carboxyl over amino; or *vice versa*, as the case may be as shown in the last section. A continuation of the titration to a final end-point in the presence of formol then shows total carboxyl; hence by difference one estimates amino or carboxyl groups not in excess (*i.e.* carboxyl or amino groups equally balanced in number). Results of analysis are given in Table B.

Results.—The values found agree with the theoretical calculation, within the small experimental error and confirm the preceding section.

5. NH_2 Determined by Titrating to a Highly Acid End-Point in Absence of Water.

In my first paper on amino acid titrations, a method for the volumetric estimation of amino groups was devised, according to which they were titrated with standard acid to a highly acid end-point; *e.g.*, less than pH 0.4. It was then shown that the sharpness of the end-point, in other words the accuracy of the method, was limited by the quantity of water present as solute in the titrated solution. Therefore to obtain optimal conditions it was directed that the test substance should be made up with as little water as possible and that the standard acid should also be as concentrated as convenient. Recently I have been experimenting along the lines of reducing the effective water concentration by such means as titrating in the presence of large amounts of glucose,

[18] *E g.* 20 per cent in the case of phenylalanine, 50 per cent in the case of glycyl-glycine, only 5 per cent in the case of glycine.

TABLE B.

Analytical Details.

	Specimen a, N.U.M.	Specimen b, N.U.M.	Specimen c, F.G.H.	Specimen c, F.G.H.
	gm.	*gm.*	*gm.*	*gm.*
Weight of glutathione taken	0.0304	0 0280	0.0307	0.0614
	cc.	*cc.*	*cc.*	*cc.*
Water added (CO_2-free)	1.0	1.0	1.0	1.0
Indicator added	1.0*	1.0†	1.0*	1.0*
Formalin (previously neutralized to the same indicator)	10.0	2, 3, 5, 7.5	10	10
Total carboxyl found. (*I.e.* standard NaOH required at final stage of titration)	**0.20** × N	**1.75** × 0.1 N	**0.21** × N	**0.40** × N
Total carboxyl calculated.	(Micro burette.)	(Macro burette.)	(Micro burette.)	(Micro burette.)
(*I.e.* theoretical for tripeptide of cysteine, glycine, glutamic acid)	**0.20** "	**1.8** "	**0.20** "	**0.40** "
Method sensitive to	0.01 "		0.01 "	0.01 "
Standard NaOH required in same titration in absence of formalin	0.10 "		0.11 "	0.205 "
By difference.				
Total amino groups found, standard NaOH.	**0.10** "		**0.10** "	**0.195** "
" " " calculated. (*I.e.* theoretical for tripeptide)	**0.10** "		**0.10** "	**0.20** "
Method sensitive to	0.01 "		0.01 "	0.01 "

* Brom-thymol blue.

† Phenolphthalein.

$CaCl_2$, etc. Only partial success has attended these devices. But, by titrating in the entire absence of water I have succeeded in obtaining extremely accurate readings with remarkably sensitive end-points. A sharp indicator color change occurs under the conditions described below, with the addition of less than

TABLE C.
Analytical Details.

	Specimen A. Twice separated as Cu salt, Fraction 1 (F.G.H.).	Duplicate of Specimen A.	Specimen B. Once separated as Cu salt.	Duplicate of Specimen B.	Specimen C. (N.U.M.).	Duplicate of Specimen C.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Glutathione taken.	0 0307	0.0307	0.0307	0.0307	0.0345	0.0280
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Volume of 0.1 N perchloric acid added	1.10	1.10	1.10	1.20	1.31	1.10
Volume of brilliant cresyl green (0.01 per cent in glacial acetic acid) added....	1.0	1.0	1.0	1.0	1.0	1.0
0.1 M glycine (in glacial acetic acid) required in back titration.	0.13	0.10	0.08	0.18	0.20	0.19
Blank correction	0 02	0.02	0.02	0.02	0.01	0.01
NH_2 found.	**0.95**	**0.98**	**1.00**	**1.00**	**1.10**	**0.90**
" calculated for the tripeptide formula.........................	**1.00**	**1.00**	**1.00**	**1.00**	**1.12**	**0.91**

0.01 cc. of titrant. Glacial acetic acid is used as solvent. Its property as a general solvent for bases has been known for a great many years past, and it has recently been employed by Hall and his coworkers in their important work on E.M.F. measurements.[19]

As a titrant perchloric acid in place of hydrochloric acid has been recommended by a number of recent writers (*e.g.*, Smith,[20]

[19] Hall, N. F., and Conant, J. B., *J. Am. Chem. Soc.*, **49**, 3047 (1927). Conant, J. B., and Hall, N. F., *J. Am. Chem. Soc.*, **49**, 3062 (1927). Hall, N. F., and Conant, J. B., *J. Am. Chem. Soc.*, **50**, 2367 (1928).

[20] Smith, G. F., *Chem.-Analyst*, **17**, 20 (1928).

Hall, *et al.*) and may also be used dissolved in glacial acetic acid solvent. It is prepared for me at N dilution by Messrs. British Drug Houses, London.

Since I have found that the amino acids themselves may be much less readily soluble than are their acid salts in the glacial acetic acid, I have adopted as a routine in estimating the basic groups in amino acids, peptides, and proteins, the practice of adding a slight measured excess of the standard perchloric acid and then back titrating the excess with base.

It is intended to publish elsewhere the detailed results of the method as applied to other peptides, and to proteins and amino acids.

Method.—To about 0.1 mg. equivalent of the substance, an accurately measured slight excess of 0.1 N perchloric acid (in glacial acetic acid) is added from a micro burette. When dissolved, a suitable indicator is added. (The indicator should have a pK value of about 0.) A search of the current catalogues shows that brilliant cresyl blue (Messrs. British Drug Houses) is the only suitable indicator which is at all readily available, and I have therefore adopted it for this work. Water must be rigorously excluded and I have found it easy to use a stock solution of the indicator dissolved in glacial acetic acid.

For the back titrations I have used as a base, a 0.1 M solution of glycine (pK = 2.4) dissolved in glacial acetic acid.[21] This keeps reasonably well. The titration is carried on until there occurs an extremely sharp discharge of the brilliant blue tint. The perchloric acid titer less the glycine back titer gives the amino groups present. (A small blank correction may be made for the amount of perchloric acid which is required to titrate the same volume of glacial acetic acid solvent plus the same quantity of indicator, in the absence only of the solute.) Data are given in Table C.

Results.—NH_2 "found" agrees with NH_2 "calculated" within 2 per cent (5 per cent in one case).

[21] The back titration should be carried out fairly soon after the indicator has been added, without its being left too long in contact with the highly reducing glutathione.

6. Determination of Equivalent Weight of Glutathione.

The results in Table C all agree in showing that glutathione has an equivalent weight of 307 with a possible error of about 2 per cent (*i.e.*, it lies between 301 and 313). (The figure 307 is arrived at as being the weight in gm. of glutathione which we determined to combine with 1 unit equivalent of acid, or with 2 unit equivalents of soda, the latter of course in two separate stages.) In order to obtain a more exact value for the equivalent weight, the glutathione was lastly titrated to as sensitive an end-point as could be found. The most sharp change was found to occur at about pH 6.2 (*e.g.* with brom-cresol purple); a reading which is in concordance with the relation

$$x = \frac{pK_3 - pK_2}{2}$$

where the observed pK values in question are roughly 8.8 and 3.6 respectively (x being the pH for maximal value of d.pH/dA).

Experimental Details.

0 056233 gm. glutathione (prepared by N. U. M.), plus 0 1 cc. brom-cresol purple indicator; no water, titrated from micro burette:

Required	**1.83**	cc. 0 1 N NaOH
Reading was sensitive to	0 005	" 0 1 " "
Calculated for the tripeptide	**1.83**	" 0.1 " "

Results.—The value here found leads to a minimal molecular weight for glutathione of **307** ± **0 .8**. That calculated from the formula, for a tripeptide comprising cysteine, glycine, and glutamic acid is **307**. (This may be taken of course as the actual molecular weight in water, since there is no evidence of polymerization.)

Desulfuration of the Tripeptide by Alkali in the Presence of Lead Acetate.

A number of experiments were earlier carried out on impure products, involving estimations of the percentage desulfuration at successive periods. It does not seem worth while to occupy space in this paper with these data. Their chief result was to demonstrate that their sulfur was much more unstable to alkali than that of free cysteine or cystine.

In the case of the pure tripeptide it has been thought sufficient to estimate the percentage removal of its sulfur at the end of one definite period.

The alkali chosen was, as in the earlier experiments, 0.33 N barium hydroxide. In 30 cc. of this 0.2 gm. of the substance were dissolved and 2 cc. of 20 per cent neutral lead acetate added. A stream of hydrogen was passed for a short time through the fluid and the flasks then corked air-tight. The solutions stood for 5 hours in a hot room at 37°. They were then strongly acidified with acetic acid, the lead sulfide filtered off, and thoroughly washed. The residue was then converted into lead sulfate in a crucible and weighed.

It was found that in these circumstances no less than 55 per cent of the sulfur of the tripeptide is removed in 5 hours, while similar solutions of cystine and cysteine showed in the same time no trace even of blackening. In one experiment cysteine treated as above lost only 4.5 per cent of its sulfur in 24 hours.

Results of Boiling with Water. Rate of Desulfuration and Diketopiperazine Formation.

With the less pure products a number of experiments were carried out. It appears desirable to publish the results of one of these as given in Table I. The rates of desulfuration and diketopiperazine formation during boiling with distilled water are there illustrated. In this and in most experiments of the kind the product was boiled in 2 per cent solution. The pH of such a solution is about 3.00. The product used for the experiment was in the oxidized form and happened to be one in which the original percentage of sulfur was somewhat higher than the average among preparations separated by the intermediate method. A current of air was passed through the boiling solution and led into a cylinder packed with glass beads and containing an ammoniacal solution of hydrogen peroxide. Beyond this the air passed through lead acetate solution but no blackening of this was observed. Oxidation with the peroxide was complete and the sulfur was estimated as barium sulfate. The free S, which after about the 20th hour began to accumulate in the condenser, was (after the condenser tube was dried) dissolved in CS_2 and weighed. At the end of each

period given in Table I the process was interrupted, the solution evaporated to a small bulk, the crystalline piperazine filtered off, washed with cold water, dried, and weighed. The filtrate was then returned to the flask, made up to the original volume, and the boiling continued. That the method secures considerable accuracy is suggested by a striking similarity in the figures and time

TABLE I.

Product (SS) Containing 12.00 Per Cent Sulfur (1.975 Gm. Containing 237 Mg. of S Boiled in 100 Cc. of Water).

Period No.	Time boiled.	S evolved as SH_2.	S eliminated as free S.	Total S eliminated.	S eliminated during each period in per cent of original.	Total S eliminated in per cent of original.	Amount of diketopiperazine formed.	Containing S.
	hrs.	*gm.*	*gm.*	*gm.*			*gm.*	*gm.*
1	24	0.028	0.0044	0.0324	13 5		0 2100	0.0420
2	17	0.0087	0.0155	0.0242	10 2	23 7	0.1270	0.0254
3	9	0.0048	0.0051	0 0091	4.2	27.9	0.0290	0.0058
	50	0.0415	0.0250	0 0657			0 3660 = 18.5% of original product.	0.0732 = 31.0% of total S.

Total S accounted for:

As SH_2 and free S	27.9 per cent.
In diketopiperazine	31.0 " "
	58.9 " " of S in original product.

relations displayed in experiments on the same lines in other cases of the less pure preparations.

When the same technique was applied to the pure crystalline substance, it was found, as stated earlier in the paper, that the rate of desulfuration is much slower than that displayed by the less pure materials, though that of diketopiperazine formation is almost the same. Thus in an experiment similar to that just discussed the sulfur eliminated from the pure substance in 42 hours boiling was only 12.3 per cent of the whole, instead of 23 per

cent, and no free S appeared, while the diketopiperazine formed was 20 per cent of the weight of the original material, as against 17.0 per cent (43 hours, end of second period, see Table I). This experiment was with the thiol form of the pure substance. In another experiment the disulfide form (not wholly free from desulfurized products yielded 10.8 per cent of its sulfur (of which a small part was in the form of free S) in 42 hours, and 17.2 per cent of diketopiperazine.

Anhydrides Formed during Boiling with Water.—The diglycylcystine dianhydride produced during the boiling of the disulfide form of the tripeptide with water is, like most diketopiperazines, readily crystallizable. It is soluble in boiling water, from which it separates on cooling as a magma of acicular crystals. Its solubility in cold water is 0.06 per cent. When obtained pure it melts sharply at 262° (uncorrected) with decomposition. It gives the color reactions described for diketopiperazines, but no nitroprusside reaction. It yields no trace of nitrogen when treated with nitrous acid in Van Slyke's apparatus, and no ninhydrin reaction. The reduced form of the above (glycyl-cysteine anhydride) formed on boiling the tripeptide in its thiol form, is a much more soluble substance. It is most simply prepared by reducing the disulfide form described above, and very easily by dissolving the latter in a large quantity of hot water, and precipitating the solution while still hot with $HgSO_4$. The white precipitate is suspended in a minimum of water and decomposed as usual with H_2S. The solution now contains the anhydride completely reduced, and on being evaporated *in vacuo* to a small bulk it yields well formed crystals of the pure substance. This melts sharply at 203° (uncorrected) without decomposition. It gives the diketopiperazine reactions and a strong nitroprusside reaction, yields no Van Slyke nitrogen, and gives no ninhydrin reaction. It is easily oxidized by aeration in the presence of iron, giving rise to the dithiodianhydride described above. The sulfur of each of these piperazine derivatives is removed with exceptional ease under the influence of even weak alkalies.

A number of experiments were performed with the less pure products to determine the yield of these anhydrides on boiling with water. The maximum yield was obtained at about the 60th to 70th hour and was somewhat greater when, in the case of SS

preparations, the crystalline product was separated in successive fractions; the contents of the flask were at each stage evaporated to small bulk, the crystals filtered off, washed with cold water, and the filtrate boiled for a further period. The yield only varied between 22 and 25 per cent. In two similar experiments with the pure tripeptide the yields were 20.15 and 22 per cent.

The oxidized form (the dithiodianhydride) has been frequently analyzed, always with consistent results. The following figures were given for example by a sample consisting of mixed products obtained in various experiments on the less pure preparations. It was once recrystallized before analysis.

A. 4 076 mg. required 5.15 cc. 0.01 N acid.
5.934 " :8.785 mg. $BaSO_4$.

The following data refer to a product obtained from the crystalline tripeptide.

B. 5.200 mg.: 7.170 mg. CO_2 and 2.13 mg. H_2O.
6 201 " : 9.138 " $BaSO_4$.
Nitrogen by micro-Kjeldahl, 17.58 per cent.
Found. A. N 17.70, S 20.33.
B. C 37.42, H 4.55, N 17.58, S 20.24.
Calculated for $C_{10}H_{14}N_4O_4S_2$. C 37.73, H 4.40, N 17.61, S 20.12.

The reduced form (glycine-cysteine anhydride) prepared from the pure substance has been once analyzed.

	C	H	S	N
5.163 mg.: 7.140 mg. CO_2 and 2.54 mg. H_2O	37.70	4.92		
5.100 mg. required 6.39 cc. 0.01 N acid				17.53
12.552 mg.: 18.000 mg. $BaSO_4$			19.77	
Calculated for $C_5H_8N_2O_2S$	37.50	5.00	20.00	17.50

From the products of acid hydrolysis of the anhydrides pure cystine and glycine have been readily obtained; the circumstance that the cysteine or cystine is completely precipitated by the acid $HgSO_4$ reagent, and the glycine not at all, makes the separation easy.

Other Results of Boiling with Water.—Identification of the residual products presents difficulties because the decomposition is complex. Useful experience in dealing with such residual products was obtained from numerous observations on the earlier prepara-

tions and certain points of interest have been established. One experiment with the pure crystalline product need alone be reported however, as the general indications have been the same in all cases.

4 gm. of this, in thiol form, were dissolved in 150 cc. of water, and the solution boiled for 50 hours. The evolution of H_2S was continuous throughout. The solution when cool was precipitated with mercuric sulfate, which removes the whole of the diketopiperazine and any material still associated with cysteine. From the filtrate excess of mercury was removed as sulfide and the sulfuric acid as barium sulfate. The solution was then evaporated to a thin syrup and allowed to stand. A crystalline fraction separated (0.141 gm.) which when once recrystallized analyzed as pure glutamic acid. Another small fraction was next removed (22 mg.) which contained 9.55 per cent N and was also pure glutamic acid. From this stage onwards the solution began to deposit long silky prismatic needles which separated very slowly. Three successive fractions were removed after long standing at each stage. All of these had exactly similar characters, and gave a nitrogen content of 10.80, 10.54, and 10.75 per cent, respectively (pyrrolidonecarboxylic acid, 10.85 per cent). The residue was next extracted with absolute alcohol and from the extract further separation of the silky needles occurred, the product containing nitrogen 10.75 per cent. Successive fractions were found to have a melting point of 160° which remained unchanged on being mixed with pure pyrrolidonecarboxylic acid, and all the fractions in question undoubtedly consisted of that substance.[8] They gave no ninhydrin reaction until after they had been boiled with HCl. The concordant data for carbon and hydrogen appear below.

In all 0.78 gm. of the pyrrolidone acid was isolated, equal to 0.90 gm. of glutamic acid; amounting, with that isolated as such, to 1.06 gm. of the latter.

Analysis of pyrrolidone fraction.

	C	H	N
5.243 mg.: 8.890 mg. CO_2 and 2.68 mg. H_2O	46.24	5.68	
Mean of micro-Kjeldahl estimation			10.71
Calculated for $C_5H_7O_3N$	46.51	5.42	10.85

[8] That free glutamic acid when boiled with water is readily converted into pyrrolidonecarboxylic acid has been shown by Foreman (Foreman, F. W., *Biochem. J.*, 8, 463 (1914)).

The mercury precipitate mentioned above was decomposed in the usual manner and the solution obtained, after being freed from H_2S and H_2SO_4, was aerated at pH 7.5 in the presence of a trace of iron. Crystals of the piperazine compound separated which were filtered off (weight 0.43 gm.; N 17.61, S 20.44 per cent). The barium used to adjust pH was then exactly removed and the solution evaporated to dryness. The residue was extracted with cold hydrochloric acid in which the piperazine derivative is but little soluble. The acid extract contained a very small quantity of free cystine, identified by its hexagonal crystals. The anhydride left behind was not quite free from admixture, but after one recrystallization it was pure, and weighed 0.32 gm. The syrupy mother liquor from the pyrrolidonecarboxylic acid (mercury filtrate) and those from the diketopiperazine (mercury precipitate) were separately hydrolyzed with HCl. From the former 0.34 gm. of pure glutamic acid was separated. It is uncertain whether this was still in combination before the acid hydrolysis or in the form of the pyrrolidone acid which had failed to crystallyze. A very small amount of glycine was identified as picrate. From the latter 0.12 gm. of pure glycine and 0.19 gm. of pure cystine were obtained, but no trace of glutamic acid. Final mother liquors contained products derived from decomposition during the boiling with water. These could not be identified.

As the mercury precipitates all products still containing sulfur, it is certain that after the tripeptide had been boiled with water no glutamic acid remained attached to cysteine. While the greater part of this amino acid was undoubtedly free, a small amount may have remained in association with desulfurized cysteine, though this was not proved. The greater part of the glycine which had escaped diketopiperazine formation would seem to remain in association with cysteine.

Evidence Suggesting That There Is Some Destruction of Carbon Chains during Boiling with Water.

In the case of two of the earlier preparations (one SS and one SH) an inlet tube was passed through the condenser into the boiling flask and CO_2-free air passed with positive pressure through an outlet tube into standard barium hydroxide solution. (The apparatus was made completely air-tight.) In each case after a few

hours the baryta became cloudy and in the course of 6 hours a considerable precipitate formed. Titration indicated that in both cases after this relatively short period some 3 per cent of the total carbon of the product had appeared as CO_2.

To obtain more definite evidence a product (SS) was boiled for 50 hours and the carbon estimated in the original and in the residue obtained after boiling. The absence of any error due to manipulation or incomplete drying, etc., was proved by the figures obtained for nitrogen. It was found that the results could be made most accurate by working on a relatively small scale with compact apparatus.

0.5 gm. of the product of which the percentage composition was known was boiled, as stated, for 50 hours.

The residue after boiling, when dried to constant weight, weighed 0.4068 gm., showing therefore a total loss of 0.0932 gm., or 18.5 per cent. Of this loss part of course was due to the removal of sulfur and to anhydride formation, but that there was also loss of carbon is shown by the following data:

	Original.	Residue.
C	37.65	39.58
H	5.80	4.90
N	11.55	13.53

$0.5 \times 37.65 = 0.188$ carbon originally present; $0.4068 \times 39.58 = 0.161$ carbon in the residue. There was therefore a loss of carbon $= 0.188 - 0.161 = 0.027$ gm. This is equal to 14.3 per cent of the carbon in the original product.

On the other hand the nitrogen content of the original product and that of the residue were, within the limits of experimental error, identical. $0.5 \times 11.55 = 0.0575$ N; $0.4068 \times 13.53 = 0.0550$ N. There is thus an apparent loss of nitrogen of 2.5 mg. only, or 0.5 per cent of the original, showing therefore that there was no appreciable loss due to the manipulation.

These results were obtained with care and were reproduced in the case of other preparations. As, however, similar experiments have not been made upon the pure product, it is not proposed to insist upon them. It is extremely probably however that the decomposition induced by boiling with water extends to the carbon structures of the tripeptide.

Oxidation of the Thiol Product by Aeration.

1 gm. of a crystalline preparation (A) was dissolved in 30 cc. of water and barium hydroxide added to make the pH 7.6. After the addition of a trace of ferrous sulfate air was passed through the solution until it no longer gave a nitroprusside reaction (5 hours). The barium was then exactly removed and the solution evaporated *in vacuo* at 40°. The residue was taken two or three times to dryness with alcohol until completely dehydrated. It was a white friable powder and was dried to constant weight *in vacuo* over P_2O_5 at 50°.

A second preparation (B) was treated in a similar manner.

The oxidized products yielded the following figures on analysis.

A. C 39.51, H 6.36, N 12.07, S 9.52.
B. " 39.89, " 6.98, " 11.99, " 9.60.

The nitrogen and sulfur values are thus considerably below those proper to the disulfide form of the tripeptide (N 13.68, S 10.42).

The product (A) was dissolved, and the solution precipitated with mercuric sulfate. The precipitate on decomposition with H_2S (which reduces the product) yielded a solution which in the presence of 0.5 N sulfuric acid gave the typical cuprous salt of the thiol compound, and from this the latter (0.7 gm.) was obtained crystalline and pure. While the greater part of the oxidized product was therefore in the disulfide form, a portion had suffered loss of N and S. For a discussion of these results see p. 276.

Concluding Remarks.

The evidence that the crystalline substance, of which the isolation has been described, has the constitution of a tripeptide seems to be so conclusive that I have not hesitated to speak of it as such throughout this paper. The analytical data, the evidence for its equivalent weight, the results of titrating its amino and carboxyl groups, and the yield of the three amino acids upon complete hydrolysis, all unite in support of this constitution.

Nevertheless aspects of its behavior have been described which may raise at least some measure of doubt concerning the linkages involved in its structure; a question as to whether they are normal amide peptide linkages. So difficult is it however to picture likely

alternatives for these linkages that an explanation of the exceptional instablity displayed by the substance may rather be sought in the nature of its constituent amino acids themselves. The glutamic acid determines the presence of two free carboxyl groups and the consequent high acidity of aqueous solutions. The inherent reactivity of the thiol group may well contribute to instability, and the simultaneous presence of the extra carboxyl and thiol groups, especially characteristic of the substance, may be sufficient to determine its behavior when boiled with water.[9]

The present paper, with full intention, has followed lines that are merely descriptive. It is certain that conclusive views concerning the constitution of the newly isolated compound must await the results of synthetic studies, and these, owing to the apparent difficulties experienced in the preparation of cysteinyl derivatives, may prove troublesome. An explanation of the behavior of the compound moreover may call for comparison with related synthetic cysteine peptides of a kind not yet available. In the following paper by Pirie and Pinhey certain conclusions as to its constitution are based upon the results of electrometric titrations. These, though of real interest, must I feel be regarded as strictly provisional.

The relatively large amount of the tripeptide found in the yeast cell indicates that it is a normal cell constituent of some prominence, and is certainly no accidental fraction of ordinary intracellular protein breakdown. That it is also present in red blood corpuscles, so remote metabolically from the yeast cell, is a further suggestion for this, and also perhaps for the general significance of

[9] M. Bergmann and his coworkers have shown that in the case of dipeptides in which serine is one member, acidity in solution tends to shift the acyl group of the second member from an amide linkage derived from the NH_2 group of the serine to an ester linkage derived from the OH group. Alkalinity in solution induces the opposite change. An intermediate stage involves the formation of an oxazolone ring with the acyl carbon attached to both nitrogen and oxygen (Bergmann, M., and Miekeley, A., *Z. physiol. Chem.*, **140**, 128 (1924). Bergmann, *Naturwissenschaften*, **12**, 1155 (1924)). If it may be supposed that the SH group of cysteine functions in a manner analogous with the OH group of serine, a thioxy ester linkage may, under conditions, be established in the tripeptide, and its presence may determine aspects of the behavior of this substance.

its presence. An attempt to identify it in other tissues is already in progress.

Any who may undertake its isolation will find that the method described is extremely easy to carry out. They may feel I think that the unusually high selectivity displayed by cuprous copper as an agent for separation has an interest of its own; and they will certainly realize that the tripeptide is to be obtained crystalline with an ease seldom found in dealing with compounds of its class.

The question perhaps arises as to whether the name glutathione should be continued as a label for the tripeptide. Whatever propriety it may have had when attached (as supposed) to a compound containing only glutamic acid and cysteine is lessened under the circumstance. Like other names of the kind however it was and must remain a mere label, and some convenience may be served in retaining it. There are chapters in the literature of glutathione which are not falsified by the circumstance that its constitution is other than was thought, and on the assumption that the literature may increase it may be well to avoid a breach of continuity.

I have to acknowledge the skilful assistance of E. J. Morgan in all manipulative operations. To the Medical Research Council my thanks are due for generous financial support.

SUMMARY.

A rapid and easy method is described for separating from yeast a pure crystalline substance with the characters of a tripeptide. It yields on hydrolysis glycine, glutamic acid, and cysteine, and its percentage analysis corresponds exactly with the composition of a tripeptide containing these three amino acids.

The yield from yeast is 1 gm. per kilo and upwards. The substance is present in red blood corpuscles.

Acidimetric titration methods have been applied (by L. J. Harris to the determination (1) of the NH_2 and COOH groups, and (2) of the minimal molecular weight. The values so found agree with those calculated for a tripeptide of the above constitution within the experimental error of 2 per cent when the routine methods generally applicable to amino acids, polypeptides,

etc., were used; or less (*e.g.* within 0.3 per cent) when special refinements were adopted.

The substance is unstable. On boiling with pure distilled water for instance it yields the diketopiperazine of cysteine and glycine, together with free glutamic acid; but also slowly undergoes a more deep seated decomposition.

The tripeptide has been shown to constitute a large proportion of the preparations of glutathione made by earlier methods. The description of that substance as a dipeptide was therefore erroneous.

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THE TITRATION CURVE OF GLUTATHIONE.

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During the past few years considerable advances have been made, by Harris and others, in the interpretation of titration curves of amino acids and peptides. It was our intention in undertaking this work simply to see whether the titration curve of the crystalline substance described by Hopkins in the accompanying paper was consistent with the view that it is a tripeptide of glutamic acid, cysteine, and glycine; *i.e.*, containing one SH, one NH_2, and two COOH groups. This turns out to be the case, and we have also been able to identify and measure the constants for each group.

The magnitude of the titration constants of a substance may in many cases serve in addition as a valuable guide in the elucidation of its detailed structure (Simms and others). We fully appreciate however that there is so little information concerning the peptides of dicarboxylic acids and of cysteine that certain conclusions drawn, in this paper, under this second heading must be considered strictly provisional.

EXPERIMENTAL.

Glass Electrode Titration.

For each curve about 25 mg. of glutathione prepared by Hopkins' (1) method were taken. The samples were weighed into a small glass tube, open at each end, and then tipped out quantitatively into the cup of a glass electrode of the type described by Kerridge (2). A few minutes shaking sufficed to dissolve the glutathione after addition of 0.8 cc. of water or dilute formaldehyde solution. The actual volumes of normal acid and alkali used were reduced to the common basis of 30.7 mg. of reduced gluta-

thione, thus making 0.1 cc. correspond to 1 equivalent of reduced glutathione.

The technique of pH determination differed from the standard procedure described by Kerridge in two points. No paraffin was used. The glass electrode and its connections, including the calomel electrodes, was in an air bath at 22° (a few degrees above room temperature). Under these conditions moisture leaks are avoided without the use of paraffin. The same KCl contact was used during the series of determinations on any sample. That the possible diffusion of KCl around the glass cap does not affect the accuracy of the pH values is definitely shown by the good agreement obtained between the glass and hydrogen electrode determinations. The KCl used was 3.5 N.

Potassium hydrogen phthalate was used as the standard for the determinations of pH. This was the British Drug Houses "A. R." quality and its pH was checked by direct measurement with the hydrogen electrode against a saturated calomel half-cell, the value for the latter being accepted as given by Clark (3). As a further check, we compare the phthalate against the half neutralized acetic acid used as standard in the hydrogen electrode determinations, and against a McIlvaine disodium phosphate-citric acid buffer.

Hydrogen Electrode Titration.

As in the case of the glass electrode solid glutathione was weighed, put into the titration vessel, and then dissolved in water or formaldehyde. A glass tube 5 cm. long and 1 cm. in diameter, fused at its lower end to a capillary U-tube, was used as the titration vessel. The hydrogen was introduced through this capillary and so kept the liquid effectively stirred. A platinized platinum wire electrode and KCl agar bridge were held from above against the inner wall of the titration vessel, leaving ample room for the burette. With this apparatus very steady readings can be obtained on 0.5 cc. of fluid. The standard used was another hydrogen electrode in a standard buffer solution.

Hydrogen electrode determinations were possible only for reduced glutathione in water since formaldehyde is blown out of the solution by the current of hydrogen. As the hydrogen and glass electrode curves agree perfectly on the alkaline side of the isoelec-

tric point, we assume that the readings of the glass electrode in this range are also reliable for systems containing formaldehyde. On the acid side of the isoelectric point however, for systems containing reduced glutathione and hydrochloric acid, the glass electrode determinations were consistently 0.2 pH lower than the corresponding readings of the hydrogen electrode. This has been confirmed by determinations with both electrodes on the same solution, when this contains reduced glutathione and HCl and has a pH in the neighborhood of 2. This effect is not due simply to the low pH of the solution since the glass and hydrogen electrodes agree perfectly in this range when pure sulfuric acid solutions are used or, as Hughes has shown (4), hydrochloric acid. With 0.1 M glycine and HCl we found a similar discrepancy between the two electrodes, of about 0.08 pH, in the range pH 2.5 to 1.5. We have therefore added 0.2 pH to all values determined by the glass electrode on the acid side of the isoelectric point.

All determinations were made at 22°.

Quinhydrone cannot be used with glutathione. This is probably due to the upsetting of the quinone : hydroquinone equilibrium by an oxidation-reduction system.

Normal HCl and CO_2-free NaOH, in 1 cc. standard burettes divided in 0.01 cc., were used. The burette had fixed to it a very fine glass tip, waxed at the point; this was immersed in the solution in the titration vessel after each addition of acid or alkali.

Blank correction curves on water and 0.4 per cent formaldehyde were determined. In both cases the correction is negligible up to pH 11 on the alkaline side. On the acid side it becomes considerably below pH 2, and is approximately the same for water and formaldehyde. The values used in drawing the curves have been corrected by subtraction of the water or formol blank from the volume of acid added. With this correction, the curves are in good agreement with the theoretical curve, calculated by the Henderson-Hasselbalch equation, as far as pH 1.7.

With the glass electrode we have determined the titration curve of impure oxidized glutathione in water and in 0.4 per cent formaldehyde. The sample used was prepared by atmospheric oxidation of pure reduced glutathione neutralized with baryta; its sulfur and nitrogen contents were respectively 92 and 85 per

TABLE I.

Reduced Glutathione; Determinations with the Hydrogen Electrode.

Observed pH.	Volume of N HCl, corrected for acid blank.	Observed pH.	Volume of N HCl, corrected for acid blank.
	cc.		cc.
2.85	0.000	2.85	0.000
2.61	0.013	2.65	0.012
2.40	0.030	2.42	0.028
2 19	0 045	2.16	0.040
1.99	0.062	2.03	0.054
1.81	0.074	1.89	0.066
1.66	0.085	1.73	0.072
1 51	0.097	1.57	0.092

Observed pH.	Volume of N NaOH.
2.85	0.000
3.09	0.017
3.32	0.033
3.55	0.046
3.80	0.065
4.08	0.077
4.54	0.094
7.42	0.107
8.19	0.122
8.51	0.138
8.74	0.154
8.92	0.166
9.08	0.183
9.27	0.203
9.41	0.218
9.51	0.233
9.74	0.254
9.96	0.272
10.24	0.288
10.52	0.303
11.33	0.315
12.43	0.336

Corrected pH.	Volume of N HCl, corrected for acid blank.
	cc.
2.79	0.000
2.44	0.023
2.01	0.055
1.57	0.087

TABLE I—*Continued.*

Corrected pH.	Volume of N HCl, corrected for acid blank.
	cc.
2.84	0.000
2.42	0.009
2.30	0.023
2.07	0.038
1.80	0.058
1.63	0.075
1.49	0.089
1.37	0.102

Observed pH.	Volume of N NaOH.	Observed pH.	Volume of N NaOH.
	cc.		*cc.*
2.85	0.000	2.83	0.000
3.22	0.023	3.29	0.028
3 75	0.058	3.69	0.057
4 36	0.085	4.22	0.080
8.56	0.145	4.47	0.089
8.97	0 178	5.20	0.096
9 25	0.209	8.00	0.120
9.57	0.245	8.30	0.131
10.07	0.278	8.78	0.164
10.33	0.289	9.39	0.224
10 46	0 302	11.23	0 313
11 04	0 322		

Reduced glutathione in 0.1 per cent formaldehyde.		Reduced glutathione in 0.2 per cent formaldehyde.	
Observed pH.	Volume of N NaOH.	Observed pH.	Volume of N NaOH.
	cc.		*cc.*
2.83	0.000	2.80	0.000
4.09	0.075	3.23	0.028
8.64	0.154	3.77	0.062
9.42	0.212	4.11	0.081
9.71	0.249	4.71	0.096
10.38	0.277	6.64	0.100
11.09	0.302	7.89	0.141
		8.85	0.181
		9.06	0.19[illegible]
		9.34	0.2[illegible]
		9.90	0.2[illegible]
		11.02	0.[illegible]

TABLE I—*Concluded.*

Reduced glutathione in 0 4 per cent formaldehyde

Corrected pH	Volume of N HCl, corrected for acid blank	Corrected pH	Volume of N HCl, corrected for acid blank
	cc.		*cc.*
2 78	0 000	2 83	0 000
2 48	0 022	2 49	0 027
2 15	0 047	2 12	0 058
1 84	0 072	1 86	0 082
1 65	0 087		
1 42	0 108		

Reduced glutathione in water; determinations made with glass electrode. As has already been stated 0 2 pH has been added to the observed value on the acid side of the isoelectric point

cent of the theoretical. From the curve the molecular weight appears to be about 740, if we assume that there are two groups titrating between the isoelectric point and pH 6. This suggests that there is present at least 17 per cent of impurity. The impurity may however be responsible for the neutralization of some of the soda added in the range pH 2.86 to 6. The pK values given are therefore only approximate, not only on account of the impurity but also on account of the great overlapping in several of the dissociation ranges.

DISCUSSION.

The four pK values for reduced glutathione and the six for oxidized glutathione are determined from the curves by fitting a stencil, cut in accordance with the Henderson-Hasselbalch equation $pH = pK + \log \frac{\alpha}{1 - \alpha}$ to the experimental points and reading off the pH corresponding to 50 per cent dissociation, *i.e.* to $\alpha = 0.5$, as has been done by Harris (5).

e values obtained for titration in water are, reduced gluta- 2.12, 3.53, 8.66, 9.62; oxidized glutathione (approximate) 2.0, 2.6, 3.3, 4.0, 8.7, 9.6.

tions were also carried out in formaldehyde solutions of concentrations since Harris has shown (6) that such give a useful indication as to which pK values corre-

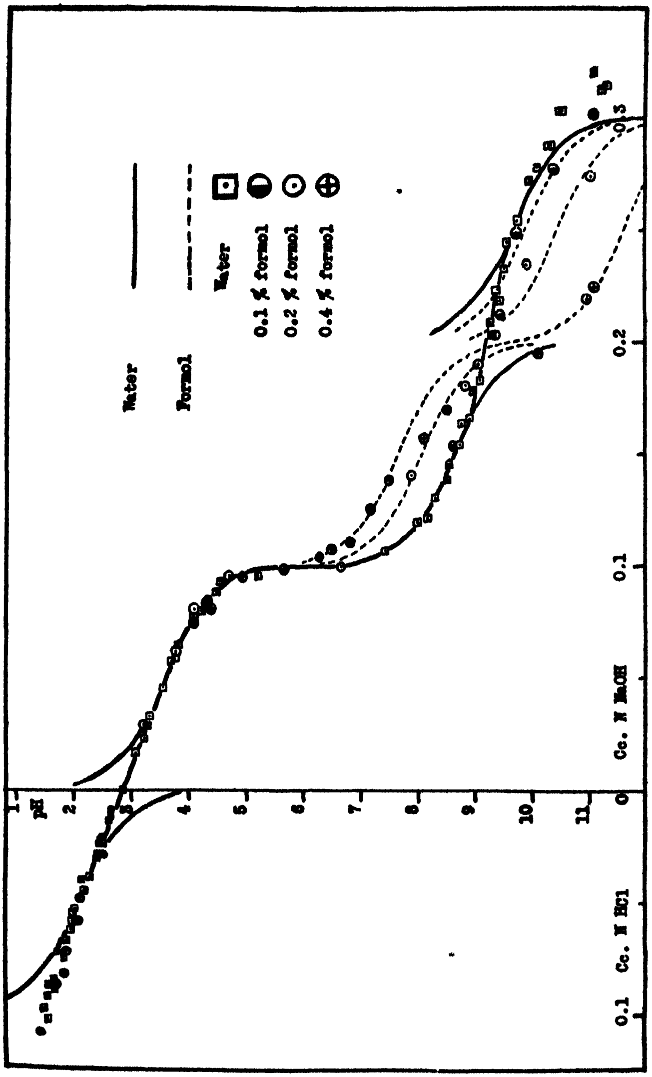

FIG. 1. Titration curve of reduced glutathione.

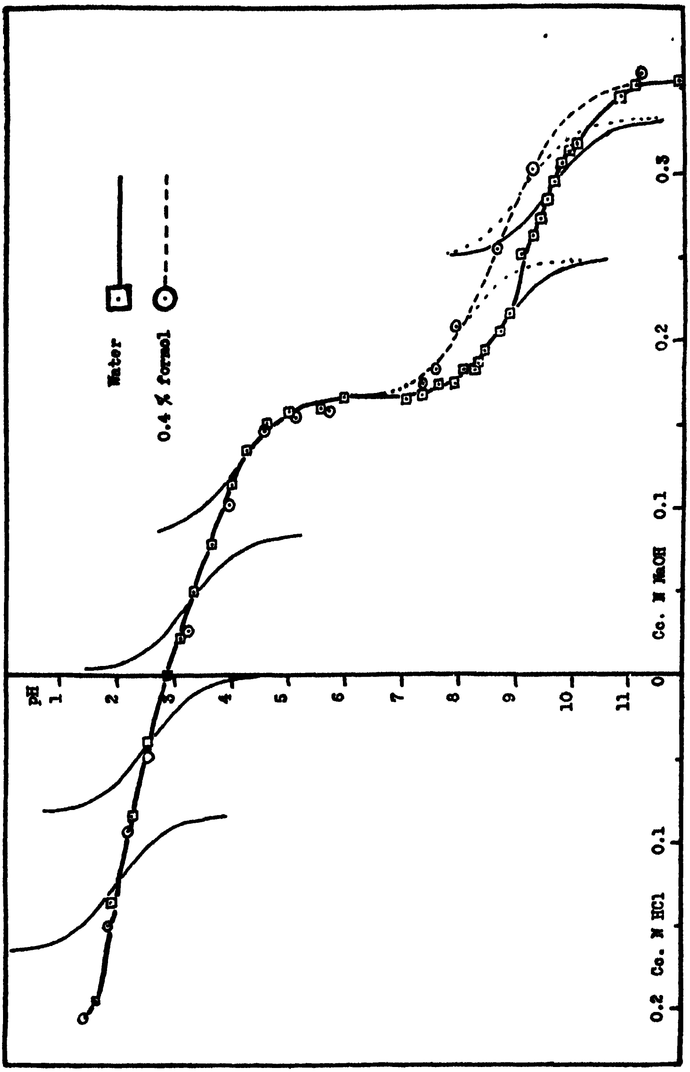

FIG. 2. Titration curve of oxidized glutathione.

spond to amino groups. Under these conditions the values at 2.12 and 3.53 for reduced glutathione and the four corresponding values for oxidized glutathione are unaltered, the other two are changed in each case. In 0.1 per cent formaldehyde there is a very slight (0.1 pH) diminution of the 8.66 value for reduced glutathione and a somewhat larger (0.2 pH) increase in that at 9.62. With 0.2 per cent formaldehyde the former is diminished by 0.6 pH and the latter increased by 0.8; with 0.4 per cent formaldehyde the diminution is 0.8 and the increase 2.2. This last value must be considered as being of the right order only, since the glass electrode becomes somewhat unreliable at such a high pH as 11 (4). The two pK values 8.7 and 9.6 of oxidized glutathione are diminished by about 0.6 pH when the titration is carried out in 0.4 per cent formaldehyde.

The equivalent weight of reduced glutathione, determined from the position of the unbuffered portion of the curve at pH 5 to 7, is in complete agreement with the theory that the molecular weight is 307 and that there is one group titrating between the isoelectric point (2.85) and pH 6. In the case of oxidized preparation of glutathione the amount of soda needed to reach pH 6 is only 83 per cent of the theoretical for a molecular weight of 612 and two groups titrating between 2.85 and 6. The cause of this discrepancy, which is apparent also in the analytical figures, is discussed by Hopkins in an accompanying paper. It is perhaps significant that the ranges of dissociation of titratable groups is sharply defined and that these are in good agreement with the Henderson-Hasselbalch equation in spite of the presumptive presence of 17 per cent of impurity.

The three pK values of cysteine and the four of cystine have been determined by Cannan and Knight (7) as 1.86, 8.14, 10.34 and <1.0, 1.5, 7.49, and 9.02 respectively. If we adopt the "zwitterion" theory of Adams and Bjerrum, it seems reasonable to assign these values as follows:

$$
\begin{array}{l}
\mathrm{SH}\ (10.34) \\
\mathrm{CH_2} \\
\mathrm{CH \cdot NH_3^+}\ (8.14) \\
\mathrm{COO^-}\ (1.86)
\end{array}
\quad \text{and} \quad
\left[\begin{array}{ll}
\mathrm{-S} & \\
\mathrm{CH_2} & \\
\mathrm{CH \cdot NH_3^+} & (7.49 \text{ and } 9.02) \\
\mathrm{COO^-} & (< 1.0 \text{ and } 1.5)
\end{array}\right]_2
$$

The value assigned to the —SH group is similar to that found by Cannan and Knight (7) in the cases of thiolactic and thioglycollic acids and the absence of a corresponding value in the oxidized compound is further confirmation of this arrangement. The values for both the carboxyl and the amino groups of cysteine are comparable with those of other amino acids although, as the authors mentioned above have pointed out, abnormally low.

Harris (5, 8) has determined the pK of glutamic acid. His values are approximately 2.2, 4.3, and 9.8; these may be assigned

$$\begin{array}{l} (4.3)\ {}^{-}OOC \cdot CH_2CH_2CH \cdot COO^{-}\ (2.2) \\ \qquad\qquad\qquad\qquad\qquad NH_3^+\ (9.8) \end{array}$$

because 2.2 and 9.8 are normal values for the carboxyl and amino groups of amino acids and 4.3 is in agreement with the value 4.8 given by Landolt and Börnstein (9) for butyric and valeric acids. Harris (5) also gives values for glycine and (6) assigns them thus, $(2.4)^{-}OOC \cdot CH_2NH_3^+\ (9.75)$.

It has been shown by Harris (5) and Simms (10) that on peptide formation there is a weakening of both the acidic and basic pK values; *i.e.*, the carboxyl pK is increased and the amino one diminished. If we assume, in the case of the carboxyl group, that this is due to the removal of the activating influence of the amino group, and *vice versa*, as in Bjerrum's theory, and if we assume also that this is a general rule for all peptides, we may attempt to assign the four pK values of reduced glutathione to their respective groups. (For the purpose of our argument it has been considered unnecessary to correct the observed pK for salt concentration or to use the more accurate γ values in applying the acid correction, as minor changes in the pK values do not affect our conclusions.)

The pK at 8.66 probably corresponds to an amino group since it is diminished in the formol titrations in a manner similar to that found by Harris (6) for the amino groups of amino acids under these conditions.

We may confidently assign the pK at 9.62 to an —SH group on account of its apparent absence in the oxidized compound and on account of its abnormal behavior with formaldehyde.[1]

[1] The reaction between formaldehyde and the —SH group appears to be an equilibrium similar to that occurring with $-NH_2$ groups since the shift of the most alkaline pK of glutathione depends on the concentration of

The pK values at 2.1
groups by analogy with c
It is therefore clear tl
bining weight of 307 and l
groups, satisfies the requ
acid, cysteine, and glycine
An attempt will now be
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necessary to wait for direct
knowledge of the titration co
positive statements can be m
The value 2.12 is probabl
glutamic acid if the adjacent a
that it corresponds to the carl
—SH pK, from a value greater t
compounds with a free carbox
opposed to the latter possibility,
the free carboxyl group is proba
first pK of H_2S from about 7.5
HS·CHR·COOH compounds. I
studied the pK of an amino grou
8.66 is therefore very high for an
jacent carboxyl group. It is, on
usual for an amino acid pK. The
than the former in view of the great
for the amino groups of amino ac
corresponds to the amino group of
jacent carboxyl group free.

---

formaldehyde even when there is present less
hyde for each molecule of glutathione. Simil.
by titrating to pH 12 with brilliant cresyl blue.
of formaldehyde 3 equivalents of soda were ne
thione or cysteine hydrochloride to pH 12 and
maldehyde only 2 were required. Between the
soda needed is a function of the formaldehyde co
is being investigated further as it does not seem
to the well known action between mercaptans
pounds of the type of mercaptol.

—NH · CH · CH₂SH
|
COOH

· CO—NH · CH₂COOH

I₂SH

·educed glutathione and the
.n the first on account of the

very satisfactory explanation
igh for an ordinary carboxyl
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acid type. If we suppose that
c acid is free, there are three

H₂CH · COOH
|
NH—RR₁

CH₂CH NH₂

CO—RR₁

I₂CH₂CH · CO—R

NH—R₁

and cysteine.

ıates to glutaric acid in which the pK
, while the second resembles γ-amino-
midt (11) found an acid pK at about 4.
third structure with the preparation by
eine diketopiperazine from glutathione.
fails therefore to provide an adequate
and until more information is available
of dicarboxylic acids we must leave the
d.

SUMMARY.

The titration curves of glutathione in water and dilute formaldehyde are given.

Reduced glutathione is shown to have the following pK values: SH 9.62, NH_2 8.66, COOH 3.53, and COOH 2.12.

From the magnitude of these constants an attempt is made to consider which are the more likely of the various possible modes of linkage of the three amino acids to form a tripeptide molecule.

We are deeply indebted to Professor Sir Frederick Gowland Hopkins, Mr. J. B. S. Haldane, and Dr. L. J. Harris for their advice and criticism in the preparation of this paper, and to Miss Gertrude Boothby for assistance during the investigation.

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BLOOD SUGAR AND AMINO ACID NITROGEN IN LACTATION IN WOMEN, WITH A NOTE ON LIPOID AND INORGANIC PHOSPHORUS.

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Within the last 5 years Widmark and his collaborators have put forward an interesting aspect of carbohydrate metabolism. Observing from their figures a difference between the level of blood sugar of lactating and non-lactating cows, Widmark and Carlens (1) advanced the view that the lower level found in the former was brought about by the removal of glucose from the blood stream through the activity of the mammary gland in its formation of lactose. Moreover, averaging the blood sugar determinations on a number of individual cows, producing differing amounts of milk, they found a progressively lower blood sugar with an increasing degree of lactation. "In diesen Bestimmungen kann man einen gewissen Zusammenhang zwischen Milckmenge und Blutzuckergehalt bemerken: je intensiver die Lactation desto niedringer ist der Blutzuckergehalt." They calculate that a cow yielding 28 kilos of milk daily uses 60 gm. of glucose an hour, and is under the necessity of renewing the circulating blood glucose every 15 minutes. Carlens and Krestownikoff (2) observed a lowering of the blood sugar during the process of milking in the cow, though they did not find an analogous phenomenon in goats. An extension of the above views led Widmark and Carlens (3) to conclude that in heavy milking cows the blood sugar might be lowered to such an extent that hypoglycemic symptoms would supervene. This condition they found in "milk fever" or "parturient paresis in cattle." Such cows showed a blood sugar of around 30 to 40 mg. per 100 cc. and recovered from their symptoms on intravenous administration of glucose solutions. The similarity between the symptoms of milk fever in cattle and the hypoglycemic symptoms following insulin injection in rabbits had been previously noted by an unknown Canadian veterinarian (4). Widmark and Carlens (5) produced insulin hypoglycemia in cows and found a great similarity of symptoms with those of milk fever. Auger (6), by slightly different methods confirmed the results of Widmark, both in the lowering of blood sugar brought about by lactation, and in the effectiveness of glucose injections in milk fever. He claimed a similar lowering of blood sugar in lactation in goats. Maguire (7), apparently not knowing of

Widmark's work, found low blood sugar values of 30 to 40 mg in cows with milk fever. He apparently assumed a blood sugar level in normal cows of 80 to 120 mg. per 100 cc. of blood, and attributed the lowering found in milk fever to a disturbance of endocrine function.

Neither the views nor the facts alleged by Widmark and Carlens, have escaped challenge. All recent investigators find a low blood sugar in milking cows. Their averages range from 48 to 77 mg. per 100 cc. A general average from all the figures we have collected. is 59 mg. on 215 determinations, ranging from 33 to 95 mg. in individual estimations (1) (7-14). The average of Widmark and Carlens is 57 mg. They state 85 mg. as the average blood sugar in heifers and dry cows. Scheunert and Pelchrzim (8), Moussu and Moussu (11), Andejewa, Prowatorowa, Sawitsch, and Thal (12), and Hayden and Fish (13) find no difference in the blood sugar level between the dry and milking states. The much more comprehensive figures of Schwarz and Schwarz and Mezler-Andelburg (14) show a difference between the lactating cow and non-milking cattle. The former show an average blood sugar of 63 mg. in contrast with 82 mg. in the latter. While this confirms the figures of Widmark and Carlens, the authors were unable to find any correlation between the amount of milk production and the lowering of the blood sugar. Hayden and Sholl (15) and Hayden (16) claim a hyperglycemia rather than a hypoglycemia in milk fever. Nor is clinical opinion unanimous on the efficacy of intravenous glucose solutions in the treatment of milk fever (17).

Amino acid N is reported as low in milking cows. Cary (18) found it rarely to exceed 3 0 mg. per 100 cc. of plasma. For whole blood the figures of Cary average 4.5 mg. per 100 cc. Meigs (19) suggests that owing to the big demand for glucose for the formation of lactose, and the paucity of carbohydrate reserves, the lack of glucose is compensated by an extra deaminization of amino acids, leaving a smaller supply circulating in the plasma. Compared with man the figures for amino acid N in milking cows are low. For plasma Berglund (20) gives the range in normal man as 4.3 to 6.2 mg., with an average of 5.3 mg. For whole blood, the average is 6.4 mg Compared with the non-milking cow, it is by no means so certain that the range of, and average, amino acid N in lactation are significantly lowered. For beef blood Blau's (21) figures range from 3.76 to 5.54 mg. on fourteen determinations, with an average of 4 81 mg. The one example of amino acid N in whole blood in the dry cow given by Cary is 3.99 mg., while the cow yielding 25 liters of milk daily was shown to have an amino acid N of 5.34 mg. We have come across no figures for plasma amino acid N in the dry animal. The suggestion of Meigs seems to require further data, before it can be accepted, as it seems unquestioned that plasma amino acids bear the same relation to milk proteins that blood glucose bears to lactose.

Despite the criticisms which have been made of the views of Widmark, the principle, that the amount of lactose produced by the mammary gland can affect the level of the blood glucose, is,

in our opinion, of sufficient import to make it worth while to see if an analogous phenomenon can be found in other animals. Similarly, attention should be paid to the level of amino acid N in lactation. We have consequently examined a series of women, relating the amount of milk production with the sugar, and amino acid N of the blood or plasma. The production of milk by women is, of course, on a much lower level than that of the cow. Nevertheless it should be possible to find some evidence of correlation if the views of Widmark represent a true physiological principle. Moreover we have been fortunate enough to obtain data on two women whose lactose production per unit weight is comparable with that of a high class dairy animal.

EXPERIMENTAL.

We have made determinations of blood sugar and amino acid N on the blood of puerperal women in the early stage of lactation. The blood specimens were taken on the morning of the 10th day after labor. By this time the involution of the uterus is rapidly nearing completion and milk flow is well established. The subjects remained in bed and on uniform hospital diet. All cases studied gave a history of normal pregnancy, labor, and puerperium. We used the method of Shaffer and Hartmann (22) for blood sugar determination and of Folin (23) for amino acid N; both determinations being carried out on plasma.

The milk production of our subjects was determined from the average result of the "2 hour lactation test" taken on the morning of the 10th, 11th, and 12th days post partum. The description of this test and its relation to the 24 hour output of milk has been described elsewhere (24). The results show that there is a rather wide range of normal milk production in women, varying from 10 to 40 cc. of milk per sq. m. of body surface per 2 hours. Amounts of milk below 10 cc. are definitely low, and amounts of milk over 40 cc. per sq. m. of body surface per 2 hours are definitely high. Women in the latter category could be classed as heavy milkers in the comparative sense. Such women are few and constitute only about 15 per cent of public ward cases. We examined in this way 130 cases. We have arranged our analytical results according to the lactation class. Our group of average lactating women contains over 90 subjects, making our

average values in this class reliable. The numbers in the low and high lactating classes are small. The results are shown in Table I.

Glucose.—The amount of plasma sugar varies within normal limits. The extreme variations found are 56 to 146 mg. per 100 cc. of plasma. 80 per cent of our cases, however, fall in the usual normal range of 80 to 120 mg. The general average is 95 mg. Morriss (25) gives 100 mg. and Bergsma (26) 94 mg. of sugar per 100 cc. of blood for women 7 days post partum. Only eight of our subjects showed 75 mg. or less plasma sugar and only one of these would fall into the high lactation group. There is no significant difference between the three groups of lactating women.

TABLE I.

Relation between Blood Sugar and Amino Acid N, Lipoid and Inorganic P with the Amount of Lactation in Women.

	Lactation group.		
	Low.	Average.	High.
Milk in 2 hr. lactation test per sq.m. surface, *cc*.	0–10	10–40	40+
No. of observations	16	89	20
	mg. per 100 cc. plasma	*mg. per 100 cc. plasma*	*mg. per 100 cc. plasma*
Average glucose	95	95	93
" amino acid N	5.3	5.2	5.1
" lipoid P	11.1	10.8	10.7
" inorganic P	4.0	3.9	4.0

Amino Acid N.—The figures fall within a normal range set by Folin and Berglund for adult man. Our range is from 4.44 to 7.00 mg. amino acid N per 100 cc. of plasma. 87 per cent of our figures lie within the extremely narrow range of 4.70 to 5.70 mg. of amino acid N. Of the eight subjects showing amino acid N below this range only one would be classed in the high lactation group. It is true our average results show a slightly decreasing amino acid N as the average lactation increases. The decrease, however, is so slight that at present, we do not consider it significant, especially when the small number of subjects in the low and high lactating groups, is considered.

In order to make certain that our figures for blood sugar repre-

sented the usual proportion of glucose and non-fermentable substances, and did not, by any chance, represent a low glucose value, with a proportion of lactose in the non-fermentable fraction sufficiently high to give a total sugar of normal range, we determined on a further series of women the fermentable and non-fermentable sugar in whole blood. The presence of lactose is common in the urine of lactating women and cows. It is generally

TABLE II.

Fermentable and Non-Fermentable Sugar in Whole Blood in a Series of Lactating Women.

Subject.	Post partum.	24 hr. milk output.	Sugar (as glucose).	
			Total.	Non-fermentable.
	days	*cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
S-g-s	12	135	112	25
Y-r-k	10	187	101	18
"	19		103	24
M-l-r	10	210	112	19
H-p-r	10	210	101	30
C-k-r	10	240	109	30
R-t-e	10	277	105	23
G-n-g	10	280	99	34
H-d-n	10	285	92	29
Mc-y	10	330	106	29
T-e-s	13	387	112	30
H-m-n	10	411	76	15
G-r-e	10	430	110	30
C-f-d	10	483	87	24
J-r-s	10	1130	102	23
Average			102	26

accepted as a non-threshold substance in human blood. Hayden (16) has examined the blood of five normal milking cows for lactose and reported its absence, though it is easily demonstrable after the suppression of lactation by air inflation of the udder in milk fever cows. The sugar determinations in our series were made by a modification of the Shaffer-Hartmann method. This modification was given to the Insulin Committee of the University of Toronto by Professor Shaffer, and has been in use in

their laboratories for over 2 years. It is very satisfactory for the determination of sugar in hypoglycemic conditions. Duggan and Scott (27) and DeLong (28) have pointed out the inhibiting effect of iodide on the reducing power of sugar towards the Shaffer-Hartmann reagent. The new reagent is made up as described in the original paper of Shaffer and Hartmann with the omission of the iodide. This is made separately as a 1 per cent solution. The sugar solution, or Folin-Wu filtrate, and the copper reagent are mixed and heated in a boiling water bath exactly as described by the original authors. The solution is then cooled at 30°, 2.0 cc. of iodide solution added, followed by 2.0 cc. of N H_2SO_4. The titration by 0.005 N sodium thiosulfate is as described by

TABLE III.

Milk Output and Blood Sugar and Amino Acid N in the Six Highest Lactating Women of Our Series.

Subject.	24 hr. milk output.	Plasma sugar.	Amino acid N.
	cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
K-k	560	86	5.14
O-l-v	480	88	5.09
Y-l	465	99	4.90
G-l-n	453	99	5.00
H-r-f	950	99	4.82
J-r-s	1200	102*	

* Whole blood.

Shaffer and Hartmann. The method is standardized against solutions of pure glucose. The new reagent will determine 10 mg. of glucose per 100 cc. of original blood. It is thus well adapted to the determination of fermentable and non-fermentable sugar in blood. For the fermentation of glucose in the Folin-Wu filtrate we used the method of Folin and Svedberg (29) or that of Somogyi (30), without materially affecting the results.

The results are shown in Table II. The general average of total sugar is 102 mg. compared with 95 mg. per 100 cc. of plasma. The non-fermentable fraction varies from 15 to 30 mg., with an average of 26 mg. per 100 cc. of blood, expressed as glucose. This corresponds to the usual range and average, nor does there appear any evidence of an increasing non-fermentable fraction with

increasing lactation. It may be concluded that in general, the blood sugar and amino acid N in women are unaffected by the degree of lactation.

Finally we record the milk output and the blood analyses of two women of exceptionally high lactating power. In order to emphasize the exceptional power of the subjects H-r-f and J-r-s we have included in Table III similar records of the four next high lactating women. The two exceptional subjects have more than twice the output of any of the other four. Compared with the ordinary lactating woman their milk output is nearly 4 times as great. The blood sugar of these two subjects and the amino acid N is normal. Moreover in subject J-r-s we had a further opportunity of study 6 weeks post partum. The amount of milk (as judged from the records of the premature baby nursery, and our own measurements) remained at the same average level of 1200 cc. a day. The blood sugar was 108 mg. per 100 cc. of whole blood, with a value for the non-fermentable fraction of 28 mg.

DISCUSSION.

While it is abundantly clear that the level of blood sugar and amino acid N in women remains at the normal non-lactating level and is not affected even by high milk production, before such results can have any bearing on the views of Widmark it must be shown that the milk output, or the lactose production, of the two classes can be comparable. The milk production of heavy milking cows may well be so great as to constitute a pathological phenomenon, even in those animals in which it is not so great as to give rise to the symptoms of milk fever.

First we have calculated the milk production of our two highest lactating subjects on the basis of a similar weight to the dairy cow, and compared the calculated output with the figures as given by Widmark and Carlens (1). Our subjects (H-r-f and J-r-s) weighed 48 and 52 kilos respectively and on the basis of the average weights of dairy cows, as shown in Table IV, their weights might vary from one-eighth to one-twelfth of the dairy animal, depending upon the breed with which the comparison is made. (Widmark and Carlens do not state the weights of their experimental animals.) On such a supposition our two subjects would produce an amount of milk varying from 8 to 15 kilos daily. Such a

milk production according to Widmark and Carlens would produce a lowering of the blood sugar, from 85 mg. to 57 mg. Proportionately our two subjects should have shown a blood sugar value of around 67 mg. instead of the actual figures of 99 and 102 mg.

Secondly we have calculated the lactose production of our two subjects and compared it with that obtained from the data

TABLE IV.

Comparing Lactose Production of 2 High Lactating Women and Averages of High Lactating Cows.

	Average weight.	Average milk production of mature cows qualified in Record of Performance 1924–28.	Average milk production per day.*	Average milk production per day.†	Average milk production per day per 100 kilos body weight.	Lactose production per day per 100 kilos body weight.‡
	kg.	*kg.*	*kg.*	*liters*	*liters*	*gm.*
Cow.						
Jersey	409	4545	13	12.6	3.1	155
Guernsey	500	5000	14.3	13.8	2.7	135
Ayrshire	500	5227	14.9	14.4	2.9	145
Holstein	636	7670	21.9	21.2	3.3	165
Woman.						
H-r-f	48		0.96	0.93	1.9	133
J-r-s	52		1.24	1.2	2.3	161

* The average milk production of mature cows qualifying in the Canadian Record of Performance is for one lactation period of 365 days. A few of the cows did not reach 365 days in one lactation period, though the milk production was sufficiently high to enable them to qualify. We have taken 350 days as the average lactation period.

† Average specific gravity of whole milk 1089.

‡ Average lactose content of cow's milk, 5.0 per cent. Average lactose content of woman's milk, 7.0 per cent.

supplied to us by the Department of Agriculture of the Dominion of Canada. The data consist of the average milk production of four breeds of cow over a number of years obtained from animals qualifying for the Canadian Record of Performance at Ottawa. The calculations are shown in Table IV. On the common basis of 100 kilos of body weight subjects H-r-f and J-r-s are producing as much lactose as dairy animals of sufficient caliber to qualify in the Canadian Record of Performance.

From the above it is clear that woman can produce large amounts of milk and amounts of lactose equivalent to the production of heavy milking cows without affecting the level of the blood sugar or the amino acid N. The control of the amount of those substances circulating in the blood lies in internal secretion rather than in the output requirement.

Note on Lipoid and Inorganic Phosphorus.

In Table I are also included our average figures for lipoid and inorganic P. Both determinations were made on the plasma. Lipoid P was determined by extraction of the plasma according to the method of Bloor (31), removal of the mixture of alcohol and ether by evaporation, and then ashing the residue according to the method of Fiske and Subbarow (32) as outlined for total P of blood. The method gave figures slightly higher than that of Whitehorn (33). Inorganic P was determined by the method of Fiske and Subbarow. Meigs (19) cited the evidence which had led him and Blatherwick and Cary to believe that the milk fat was formed from lipid substances circulating in the plasma. Correlated with this was an increased inorganic P in the plasma returning from the mammary gland. Our figures show no connection between the lactation output and circulating lipoid P and inorganic P. Our average value (3.9 mg.) is slightly higher than those generally reported for inorganic P in lactation in women (34). 93 per cent of our figures lie in the range 3.3 to 4.5 mg. Fish (35) has recently found a lowered average inorganic P in serum of milking cows compared with the dry animal. Our figures support the opposite conclusion in women (36). The variation in lipoid P is more pronounced (8.0 to 14.8 mg.). The general average is 10.8 mg. with 62 per cent of our figures between 10 and 12 mg. Reference to serum Ca can be found in another paper (24).

SUMMARY.

Blood sugar and amino acid N are not affected by lactation in women.

The non-fermentable fraction of "blood sugar" remains within its ordinary range during lactation in women.

These statements are true, even in women of very high milk production.

Values are given for lipoid and inorganic P in plasma during lactation in women.

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THE SERUM CHOLESTEROL, LECITHIN PHOSPHORUS, AND FATTY ACIDS OF PIGEONS FED BEEF TISSUES.

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The beneficial effect of liver and kidney therapy (1) for patients with pernicious anemia is now well established. Not only do the red blood cells and hemoglobin increase to normal levels, but profound changes also occur in the lipoid metabolism at the onset of remission (2). These latter changes involve not only the lecithin phosphorus but particularly and prominently the cholesterol. At the onset of remission the cholesterol and the lecithin phosphorus in the plasma increase suddenly, concomitant with the response of the reticulocytes and before there occurs any distinct increase of the concentration of red blood cells and hemoglobin. This rise of the cholesterol occurs irrespective of the form in which the potent principle effective in pernicious anemia is fed. There is evidence that points to a relationship between this principle and the cholesterol metabolism (2, 3).

The mode of action of liver and liver extract on the hematopoietic organs and the organism as a whole is as yet little understood. The influence of feeding various beef organs on blood regeneration in pigeons with a nutritional anemia and thus partial bone marrow aplasia produced by fasting has been studied (4), in an effort to elucidate this problem. Definite differences had been obtained in the reaction of the blood and the hematopoietic organs of pigeons fed either beef liver, muscle meat, pancreas, spleen, or brain. Liver and kidneys were less favorable for the regeneration of the blood in this type of nutritional anemia.

The present paper deals with the response of the lipoids in the blood serum of the above series of pigeons fed for variable lengths of time with either mixed grain, with beef muscle, or beef organs after a period of fasting that rendered the birds anemic.

Experimental Procedure.

The preparation of the food and the method of feeding of the pigeons were the same as previously reported (4). At the end of the period of feeding, food was withdrawn for from 20 to 24 hours before the animal was killed and the blood collected. The cholesterol and the fatty acids were determined with the saponification method of Bloor, Pelkan, and Allen (5), and the lecithin phosphorus according to the method of Whitehorn (6).

The results obtained are set forth in Tables I to III.

TABLE I.

Lipoids in Stock Animals on Grain.

Pigeon No.	Initial weight.	No. of days fed.	Gain or loss of weight.	Cholesterol.	Lecithin phosphorus.	Fatty acids.
	gm.		*gm.*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*
110	304	45	+14	278	18.2	966
126	298	180	+50	147	27.2	844
127	270	180	+55	140	29.0	872
128	305	180	+45	154	33.2	908
171	300	90	+22	195	37.0	
172	231	90	+18	167	36.0	
173	278	90	+16	268		
56*	399	28	−20	500	30 0	1775
Average.				192	30.1	897

* Not included in the average because this pigeon was fed for 57 days with broiled liver before the grain diet was begun.

TABLE II.

Lipoids at End of Fast.

Pigeon No.	Initial weight.	No. of days fasted.	Weight after period of fasting.	Loss of weight.	Cholesterol.	Lecithin phosphorus.	Fatty acid.
	gm.		*gm.*	*gm.*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*
54	264	7	175	−89	70	3.4	534
46	333	12	200	−133	93	3.9	526
68	250	7	188	−62	125	9.3	710
25	254	11	131	−123	200	10.5	695
Average.					122	6.7	618

DISCUSSION.

The observations show that normal pigeons as well as pigeons first fasted and then fed on an exclusive diet of beef muscle or beef organs develop a profound change in their cholesterol metabolism with relatively little alteration in the lecithin phosphorus or fatty acids. The change was most marked after feeding beef liver, when the cholesterol in the blood serum increased on the average 4.3 times more than it did in pigeons fed mixed grain.

The influence of liver feeding on cholesterol metabolism of pigeons is further illustrated by Pigeon 56. The effect on the lipoids of 57 days of liver feeding was not eliminated even after 4 weeks of exclusive grain feeding, as is shown by the fact that at that time more than double the amount of both cholesterol and fatty acids was found in the blood serum. But kidney, pancreas, muscle, and spleen feeding also increased the serum cholesterol 3.4, 2.2, 1.9, and 1.7 times more than the feeding of grain. The residue of liver after extraction with 45 per cent ethyl alcohol gave an average increase of 3.0 times the standard. So far as can be determined, no cholesterol was extracted with 45 per cent ethyl alcohol, as evidenced by the negative cholesterol reaction of the extracting fluid after the extraction had been completed.

From an examination of Table III it is evident that the lower cholesterol content in the serum of these pigeons fed alcohol-extracted liver was not due to a decrease of consumption in so far as can be judged from the increase of the weights of the pigeons in a certain time interval. Paradoxical as it seems, feeding with broiled and dried brain decreased the cholesterol and lecithin phosphorus in the serum to below normal in spite of the fact that brain contains more lipoids than any other material fed. The consumption of brain was large, and the stools of these pigeons were voluminous and very foul, comparable in character to the stools of sprue.

The cholesterol, lecithin phosphorus, and fatty acids after a period of from 7 to 11 days of fasting were invariably low (Table II). However, at the time the blood was collected the pigeons were emaciated and moribund. Different results might be obtained after shorter periods of fasting and when the animal was in good condition.

TABLE III.

Lipoids in Blood Serum of Pigeons on Various Diets.

Pigeon No.	Initial weight.	Weight at beginning of feeding.	No. of days fed.	Gain or loss of weight.	Cholesterol.	Lecithin phosphorus.	Fatty acids.
Grain.							
	gm.	*gm.*		*gm.*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*
7	339	209	21	+85	308	26.0	815
72	295	171	26	+83	250	24.6	1363
83	278	172	30	+83	232	20.0	934
96	250	150	39	+128	246	28.6	914
14	276	177	53	+99	260	24.8	833
87	331	195	22	+206	250	21.2	882
88	360	201	37	+168	274	26.1	952
86	281	153	39	+120	236	22.5	938
Average................................					257	24.2	954
Broiled liver.							
44	323	215	5	+56	586	18.5	422
45	355	255	12	+75	667	25.2	412
9	334	208	21	+125	1001	24.8	
121	367	213	24	+127	1071	21.2	1042
27	290	215	32	+70	1501	33.7	1171
28	308	243	39	+83	1667	29.5	1145
24	223	Not fasted.	49	−6	1300	18.5	852
40	330	" "	49	+23	1126	32.5	666
Average................................					1115	25.5	815
Alcohol-extracted liver.							
111	299	180	27	+120	833	16.4	996
66	351	226	35	+121	750	22.1	952
64	410	245	38	+110	670	23.2	967
70	330	220	39	+103	735	16.4	1435
84	327	220	39	+149	735	19.2	1042
90	285	170	53	+82	937	24.8	1034
Average................................					776	20.3	1071

TABLE III—*Continued.*

Pigeon No.	Initial weight.	Weight at beginning of feeding.	No. of days fed.	Gain or loss of weight.	Cholesterol.	Lecithin phosphorus.	Fatty acids.
			Broiled kidney.				
	gm.	*gm.*		*gm.*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*	*mg. per 100 cc. serum*
52	423	312	5	+28	751	15.3	
36	396	300	14	+69	714	22.6	
39	327	226	21	+87	1608	25.4	666
34	297	194	24	+107	856	31.8	602
78	416	250	39	+206	576	25.0	894
38	332	215	41	+123	416	20.6	546
76	262	160	43	+134	833	26.7	982
94	277	173	49	+147	597	18.8	966
35	392	Not fasted.	51	−43	1224	32.4	
37	420	" "	54	+16	1200	38.4	
Average					877	25.7	776
			Broiled meat.				
20	300	179	4	+21	355	24.6	551
29	322	235	32	+60	577	37.0	
31	292	212	39	+79	612	32.7	885
18	294	197	53	+123	375	17.0	517
42	368	Not fasted.	49	+34	833	31.1	614
26	240	" "	54	+32	306	30.0	500
Average					509	28.7	613
			Broiled pancreas.				
51	279	171	25	+116	335	25.7	
53	386	274	25	+97	600	29.1	
98	350	231	39	+128	535	17.6	938
82	458	267	46	+203	892	20.2	1026
80	404	253	47	+151	597	19.6	1002
Average					592	22.4	988
			Broiled spleen.				
119	330	200	4	+21	155	13.6	882
125	414	190	20	+99	370	24.6	1289
122	408	195	25	+86	480	17.2	940
117	383	177	33	+98	624	22.8	1026
120	325	213	40	+81	658	27.2	1056
Average					457	21.1	1038

TABLE III—*Concluded.*

Pigeon No.	Initial weight.	Weight at beginning of feeding.	No. of days fed.	Gain or loss of weight.	Cholesterol.	Lecithin phosphorus.	Fatty acids.
Broiled and dried brain.							
	gm.	*gm.*		*gm.*	*mg. per 100 cc. serum*	*mg per 100 cc. serum*	*mg. per 100 cc. serum*
50	342	246	19	+28	220	13 7	
108	383	208	19	+37	135	16 8	908
49	344	221	28	+54	207	12 6	
Average					187	14.3	

The most obvious explanation for the increase of the cholesterol might seem to be that it is due to the amount of cholesterol contained in the food. Very few data concerning the cholesterol content of various beef organs are recorded in the literature. Grigaut (7) found 0.25 per cent in the fresh calves liver as contrasted with 0.28 per cent in the sweetbread, 0.35 per cent in the kidney, and 2.0 per cent in the brain, while Cooper (8) found 0.4 per cent in steer spleen. These figures, if representative, are directly opposite to what would be expected from the examination of these pigeons' blood serum.

That some regulatory mechanism of cholesterol exists in birds is suggested by work reported by Warner and Edmond (9) who found the cholesterol higher in laying than in non-laying hens and male birds. Likewise, Parhon and Parhon (10) observed that the blood cholesterol of domestic fowls was higher in the period just before laying. These latter authors concluded that the blood of birds enriches itself in cholesterol before the period of laying, and when the cholesterol is stored in the yolk there is a fall of cholesterol in the blood.

The data recorded in the present paper on the cholesterol metabolism of pigeons in relation to the feeding of various animal tissues as an exclusive food derive their interest mainly from the fact that there seems to be a correspondence between the increase of the cholesterol in the serum and decrease of blood formation (4). The question also arises whether the cholesterol level of the blood in these animals was regulated by some substance similar to or identical with the active principle effective in perni-

cious anemia which has been shown (2) to influence the cholesterol metabolism in pernicious anemia irrespective of the amount of cholesterol in the food.

CONCLUSIONS.

1. Determinations of the cholesterol, lecithin phosphorus, and fatty acids have been made on the serum of 63 pigeons fed with grain or with one of several beef organs as exclusive foods after a fasting period.

2. The greatest increase of cholesterol occurred after broiled beef liver had been fed. The order in which the other substances increased the cholesterol was kidney, residue of liver extracted with 45 per cent ethyl alcohol, pancreas, muscle meat, spleen, and grain. No striking average changes were observed in the lecithin phosphorus and fatty acids. Brain proved inadequate as a food and cholesterol and lecithin phosphorus in the blood serum were decreased.

3. There is a striking inverse relation between red blood cell and hemoglobin formation (4) and the increase of the cholesterol in the blood. Liver was the least and spleen the most favorable for regeneration of red blood cells and hemoglobin.

4. It is doubtful that the increase of cholesterol depended on the cholesterol of the food. The regulation of the cholesterol level in the blood serum of pigeons fed with animal organs may be due to some substance other than cholesterol which is present in varying amounts in animal organs.

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THE INFLUENCE OF THE INGESTION OF TRICAPROIN* ON THE BODY FAT OF THE WHITE RAT.

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It has become almost a universal practice to teach that the chemical composition of the "fat" of a normal animal can be altered as the result of the ingestion by that animal of a diet predominating in certain fatty acid radicals. That derivatives of such radicals should be found in the fatty substances of animals ingesting such radicals is a conservative corollary of this theory. While there is considerable experimental evidence in support of the corollary, an apparent exception was noted in a recent investigation by the writer (1). In that study a report was made on the nature of the "fat" deposited by white rats ingesting a diet containing the butyryl radical. The nature of the fatty substances in the tissues of these animals differed from that of animals on a diet almost devoid of preformed "fat." This was apparent from a comparison of the iodine numbers of the two types of "fat." The general rule was thus obeyed. The corollary, on the other hand, was not supported as no appreciable difference could be demonstrated between the saponification numbers of the two kinds of lipids. These facts led to the tentative conclusion that while the butyryl radical was apparently utilized to build up fat foreign to that synthesized from "fat"-free precursors, this difference was, however, not due to the incorporation in the body of the fatty acid radical fed, since such a procedure should result in the formation of a "fat" with a higher saponification number than that of the "fat" of control rats. It was necessary to make the conclusion a tentative one for the reason that the experimental ani-

* Purchase of this material was made possible through a grant from The Faculty Research Fund of the University of Michigan.

mals on this diet were apparently not normal. They failed to grow as well as the control rats and were subject to polyuria, a condition probably caused by the alkaline sodium butyrate in which form all of the butyryl radicals were added to the diet. Confirmation of this anomaly was therefore necessary. Weight was added to the writer's findings by the preliminary report of Davis (2) in which it was shown that amounts of the butyryl radical in body "fat" were independent of the ingestion of tributyrin.

The writer has thus far failed to find a derivative of butyric acid which could be used as a satisfactory component of the diet for the white rat. Tributyrin is so extremely bitter that the

TABLE I.

*Composition of Diets.**

	Diet I.	Diet II.
	per cent	*per cent*
Vegex	2	2
Salt mixture.†	5	5
Casein	20	20
Tricaproin		15
Starch	73	58

* The non-saponifiable matter obtained from 7 gm. of cod liver oil was added to each 100 gm. of the diets.

† Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

animals refused to eat a diet containing as little as 5 per cent of the fat. The present communication is a report of a study of the effects of adding a derivative of caproic acid to the diet of the white rat. The material used was tricaproin, a product purchased from the Eastman Kodak Company. Preliminary experiments showed that as much as 15 per cent of this neutral fat could be incorporated in the diet without making the food distasteful to the animals. This diet, as well as the one ingested by the control animals, is described in Table I. The experiments were carried out in a manner similar to that previously employed by the writer (1). Briefly stated, this consisted of placing young white rats weighing approximately 50 gm. on the diet and maintaining them thereon for a period of 8 weeks. At the end

of that time the animals were killed with illuminating gas, the gastrointestinal tract removed and discarded, and the "fat" isolated from the residual part of the animal by extraction, first with absolute alcohol, then with chloroform, and finally with ethyl ether. The combined extracts were then concentrated *in vacuo* to a small volume and the fatty substances removed from this concentrate by extraction with petroleum ether (b.p. 20–40°).

TABLE II.

Influence of Ingestion of Tricaproin on the Fatty Substances of the White Rat.

Rat No.	Diet.	Weight* of animal.	Total lipids.	Iodine No.	Saponification No.	Reichert-Meissl No.	Polensky No.
		gm.	per cent				
101	Fat-free.	176	14.4	69	189	0.97	0.51
102		101	13.3	69	192	1.10	0.45
103		133	9.7	67	196	1.23	0.65
104		175	18.1	71	197	1.38	0.59
105		114	12.2	66	189	1.50	0.65
106		136	10.6	66	202	1.39	0.65
107		146	9.7	66	186	1.35	0.69
108		147	19.5	71	195	1.33	0.55
Average.				68	194	1.28	0.59
201	Tricaproin.	173	19.8	57	194	0.94	0.61
202		164	11.7	62	192	1.20	0.51
203		154	10.4	58	193	1.36	0.62
204		150	12.1	62	185	1.40	0.77
205		158	16.9	59	192	0.99	0.54
206		159	10.5	61	188	1.51	0.58
207		176	21.3	62	194	1.52	0.71
Average.				59	191	1.27	0.62

* Less gastrointestinal tract.

Most of the results obtained are summarized in Table II. In that detailed summation are given the percentage of the total lipids, the weight of the material analyzed, the iodine and saponification numbers, as well as Reichert-Meissl and Polensky numbers. While it is true that there is a variation in the final weights attained by the different animals, this does not apparently play a rôle in determining the type of "fat" present in the animals

at the time they were sacrificed. This is to be noted from a comparison of the weights of the animals with the constants determined. While the iodine and saponification numbers are found to be independent of the body weight of the animals, a distinct difference is to be noted between the degree of unsaturation of the types of "fat" deposited by the control and experimental (tricaproin) animals. The average iodine number of the lipids obtained from the control rats is 68 as compared with 59 for those secured from the group ingesting the fat. Differences were likewise noted between the iodine numbers of the fatty acids in the phosphatides synthesized by each group of rats. Owing to lack of material, it was impractical to isolate the phosphatides from the fatty substances obtained from each individual rat, and a composite was therefore made of portions of extracts obtained from each group. The iodine number of the fatty acids in the phospholipids secured from the control group was found to be 107 compared with 97 for the other group.[1] These differences are significant enough to lead to the conclusion that as the result of the ingestion of tricaproin more saturated fatty acid radicals are present in the "new fat" than in the almost purely synthetic product. The average saponification number of the former is 191 as compared with 194 for the latter. Had the caproyl radical been incorporated in the tissues, then the saponification number of the lipids removed from animals ingesting Diet II should have been higher than that of the "fat" of the control animals. It is therefore evident that these results are in accordance with those previously reported for the "fats" obtained from animals ingesting the butyryl radical. While these data presumably point to the absence of abnormal amounts of fatty acid radicals of low molecular weight in the tissues of these experimental animals, stronger support in favor of that contention is to be obtained from a consideration of the data concerned with the Reichert-Meissl and Polensky numbers. The former is an index of the amounts of volatile, water-soluble fatty acids present, and the latter of the amounts of volatile, water-insoluble fatty

[1] The iodine number of the fatty acids obtained from the phosphatides of the control rats is in close agreement with the figure recently reported by Sinclair (3).

acids. Of the two, the Polensky number should be affected more by the presence of the caproyl radical than the Reichert-Meissl number, inasmuch as caproic acid, though volatile, is not very soluble in water. A mere glance at Table II shows the entire absence of evidence pointing to the presence of more of the radicals in question in the tissues of those animals ingesting them than are to be found in the fatty substances of the control rats. That these radicals are utilized is apparent from the fact that the two types of fats analyzed are different, but that they are not deposited as such follows from the data in Table II.

Fatty acids of low molecular weight are in the minority in all animal fats with the exception of butter fat, although they are undoubtedly ingested by some animals, especially man who eats butter. In this connection attention should be called to an unpublished result of the writer in which it was shown that only 2.7 gm. of volatile material could be obtained from as much as 4.5 kilos of fatty acids of human subcutaneous fat. While no data concerning the diet of the individual from which this material was obtained are available, it is almost safe to assume that butter was probably a part of the daily diet of the individual. The iodine and saponification numbers of this sample of human "fat" were like those previously reported for another sample (4).

These data and those previously reported suggest that fats like tributyrin and tricaproin are utilized by the white rat to form "new fat." This differs from results obtained by feeding material containing the myristyl, oleyl, or linoleyl radicals in that neither the butyryl nor the caproyl radicals can be detected in the fatty substances isolated from animals ingesting such radicals. Fatty acids of low molecular weight are probably utilized to synthesize saturated fatty acids with a mean molecular weight similar to that of those fatty acids synthesized from fat-free precursors.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

XI. THE INFLUENCE OF COD LIVER OIL UPON CALCIUM METABOLISM OF MILKING COWS.*

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Our earlier work as well as the work of other investigators (1, 2) has demonstrated that liberally milking cows, that is, cows producing above 35 pounds of milk per day, are very likely to be in negative lime balance even if fed the most liberal lime-containing roughage commonly used; namely, alfalfa hay. Occasionally, there have been observations showing that through the use of alfalfa hay with high producing cows calcium equilibrium has been maintained, but most of the investigations have shown exactly the reverse of this situation. Whether negative calcium balances are normal physiological conditions with these animals during the early part of their lactation is still a question, although it is manifest that with certain rations and with certain animals equilibrium or positive balances can be maintained even in the early part of lactation. How far the continuous maintenance of calcium equilibrium in the milking cow will aid in a sustained milk production is still a problem; and not until we have definite ways of maintaining calcium equilibrium during the entire period of lactation will we have an opportunity of learning whether such maintenance can aid sustained milk production.

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Our observations (3) on the effect of ultra-violet light upon dairy cows have been on the whole disappointing, and have led us to the conclusion that an application of ultra-violet light for an hour a day has practically no favorable influence upon calcium and phosphorus assimilation. In respect to phosphorus assimilation, it has been our experience that equilibrium of this element is more readily obtained with the ordinary rations than is calcium equilibrium, especially where the level of phosphorus is such as is obtained through the feeding of a grain mixture fortified with 20 to 25 per cent of wheat bran. Such a ration brings the phosphorus intake for a dairy cow (giving 40 to 50 pounds of milk per day) into the neighborhood of 1200 to 1400 gm. of P_2O_5 per week. Raising the calcium level in the ration of dairy cows to a point where the intake in gm. of CaO is practically equivalent to that of the P_2O_5 mentioned above will not insure a calcium equilibrium although the ratio of calcium to phosphorus would be approximately 1:0.5, which may be considered a favorable ratio. In all the metabolism trials we have run with dairy cows, our most successful experiences in maintaining equilibrium or positive calcium balances have been when the *animals received green grasses fortified with a calcium salt,* like calcium carbonate or bone meal. On these rations the calcium oxide intake per week was approximately 1350 gm. and the P_2O_5 per week approximately 1250 gm. with cows giving from 300 to 400 pounds of milk per week. Where marl was a supplement to the green grasses the calcium-phosphorus ratio was 1:0.57. Where bone meal was the supplement to green grasses the ratio was 1:0.86. In both cases equilibrium or positive lime balances were maintained, but we have had no such successful experiences with the dry hays, such as are ordinarily used in winter feeding. A review of all of our data as well as that of others would indicate that if more of the antirachitic vitamin could be introduced into the ration of this species, probably better utilization of calcium would follow. Apparently these animals receive all of their antirachitic vitamin from the feed and certainly there is no evidence that what they receive from the ordinarily cured hay is generally sufficient to influence favorably the calcium balance. On the other hand, it appears that occasionally a hay (our experience has been with alfalfa) is produced which has certain properties adequate to maintain calcium equilibrium in

this class of animals when the daily milk production is not over 40 pounds. The problem of hay curing in relation to its antirachitic content and its feeding value for dairy cows needs very much more study. However, to obviate any variation that may occur in the antirachitic content of hays it was decided to provide a constant source of vitamin D in the ration of dairy cows through the administration of cod liver oil.

Meigs (4) has already tried cod liver oil in the feeding of dairy cows and has found unimproved calcium utilization. In his experiments the cod liver oil was administered during the course of an experimental trial and it is entirely possible that this procedure upset the appetite of the animal, thereby bringing about unfavorable calcium assimilation. In our experiments we aimed to accustom the animals to cod liver oil administration. Two of the animals used in the metabolism work, which started in October, 1927, had received cod liver oil for more than a year. Cow 1 which freshened July 9, 1927, and was used in our experiment in October, 1927, had received daily ½ pound of cod liver oil since February 1, 1926. Cow 2 which was fresh September 9, 1927, and was also used in the metabolism experiment in October, 1927, had received ½ pound of cod liver oil daily since August 25, 1926. Cow 3 was fed the same ration as Cows 1 and 2 but without the cod liver oil. This cow was a somewhat heavier milker than either Cow 1 or Cow 2 and in consequence of this received daily 2 pounds more of alfalfa hay than the other two cows. This was done in order to adjust more equitably the calcium intake in reference to its milk production as compared with that of the other two animals.

The ration fed these animals consisted of a grain mixture composed of 35 pounds of corn-meal, 30 pounds of ground oats, 30 pounds of wheat bran, 5 pounds of oil meal, and 1 pound of common salt. This grain mixture was fed at the rate of 14 pounds per animal per day. All of the cows received in addition 25 pounds of corn silage per day. Cows 1 and 2 received 10 pounds of alfalfa hay of unknown curing history and Cow 3 received 12 pounds of the same hay. All the animals received ½ pound of marl per day and Cows 1 and 2 received ½ pound of cod liver oil daily, which was equivalent to about 1.7 per cent of the air-dried matter of the ration. Cow 3 did not receive cod liver oil. The cod liver

oil was of medicinal grade and of high vitamin D potency. Its vitamin D content was determined by its ability to protect baby chicks against rickets when constituting 1 per cent of the grain mixture of an experimental ration used in producing rickets in baby chicks. This grain mixture consisted of 97 parts of yellow corn, 2 parts of calcium carbonate, and 1 part of common salt, fed with skimmed milk *ad libitum*. At a level of 1 per cent of the grain mixture the cod liver oil was distinctly potent in producing bone calcification as shown by the silver nitrate test and the ash

TABLE I.

Potency of Cod Liver Oil Used in Metabolism Work.

Chick No.	Ash in tibia.	Silver nitrate test.	Weight of chick 6 wks old
Basal ration plus cod liver oil as 1 per cent of mash.			
	per cent		*gm.*
7211	48 9	Narrow uncalcified metaphysis.	315
7212	51 7	" " "	245
7214	48 4	" " "	260
7219	50 0	" " "	240
Basal ration without cod liver oil			
7203	37 8	Wide uncalcified metaphysis.	200
7204	37 4	" " "	205
7207	37 0	" " "	205
7208	40 4	" " "	230

analysis of the tibias of these chicks when 6 weeks of age. The data supporting this conclusion are shown in Table I.

The metabolism trial with the cows was begun on October 25, 1927, and lasted for 5 weeks. There was no trouble whatever in obtaining complete consumption of the ration by all of the animals. The animals were quiet and comfortable throughout the experiment and there was no reason to suspect that any loss of appetite or nervous condition was adversely influencing the utilization of calcium. The animals were all in the early part of their lactation period—Cow 1 being fresh July 9; Cow 2, September 9; and Cow 3, September 15. The calcium oxide intake for these animals was from 1514 to 1628 gm. per week. The P_2O_5 intake

was approximately constant at 1250 gm. per animal per week. This gives a calcium-phosphorus ratio of 1:0.49; with the calcium oxide content of the air-dried matter of the ration at approximately 1.6 per cent and the P_2O_5 as 1.1 per cent. The animals maintained their weight throughout the experiment. Cow 1 weighed 1233 pounds at the beginning and 1215 at the end; Cow

TABLE II.

Record of Calcium Balance and Milk Production of Cows 1, 2, and 3.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per wk.	Balance per day.	Milk per wk.
Cow 1. Received 0.5 pound of cod liver oil daily.								
1927	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Oct. 25–31	1351.7	5.4	185.1	1542.2	1513.9	−28.3	−4.0	301.8
Nov. 1–7	1358.8	11.0	207.4	1577.2	1513.9	−63.3	−9.0	302.5
" 8–14	1341.0	9.5	194.2	1544.7	1513.9	−30.9	−4.4	285.2
" 15–21	1438.1	10.0	216.3	1664.4	1513.9	−150.5	−21.5	265.9
" 22–28	1360.0	5.6	210.5	1576.1	1513.9	−62.2	−8.9	295.3
Cow 2. Received 0.5 pound of cod liver oil daily.								
Oct. 25–31	1303.4	4.0	205.1	1512.5	1513.9	+1.4	+0.2	332.3
Nov. 1–7	1390.8	9.1	227.2	1627.1	1513.9	−113.2	−17.7	314.7
" 8–14	1386.7	6.5	226.6	1619.8	1513.9	−105.9	−15.1	311.9
" 15–21	1389.9	5.9	214.9	1610.7	1513.9	−96.8	−13.8	295.9
" 22–28	1450.7	4.7	209.6	1665.0	1513.9	−151.1	−21.4	292.2
Cow 3. Received no cod liver oil.								
Oct. 25–31	1427.8	3.7	229.9	1661.4	1628.3	−33.1	−4.7	372.7
Nov. 1–7	1552.1	8.0	234.9	1785.0	1628.3	−156.7	−22.7	374.9
8–14	1504.8	6.1	239.2	1750.1	1628.3	−121.8	−17.4	363.4
15–21	1470.3	6.2	236.8	1703.3	1628.3	−75.0	−10.7	359.8
22–28	1479.8	11.4	220.2	1711.4	1628.3	−83.1	−11.7	359.4

2, 1143 pounds at the beginning and 1164 at the end; Cow 3, 1303 pounds at the beginning and 1266 at the end. None of the cows was bred at the time of the experiment. In Table II is given the record of the calcium balances and milk production of these animals.

It is apparent from the data presented that the cod liver oil has had no influence in improving the calcium assimilation of these animals. Cows 1 and 2 were in distinct negative balances through-

out the entire period, while Cow 3, which received no cod liver oil, was also in negative balance, but to no greater degree than the other two animals.

The question arose as to whether or not the cod liver oil and vitamin D had been absorbed from the intestinal tract. It is well known that this species has very little capacity for absorption of oils, as has been demonstrated by Mendel and Daniels (5) in attempts to color the milk with Sudan III. They found that the secretion into the milk of Sudan III dissolved in olive oil was decidedly faint in the case of the cow as compared with the goat.

TABLE III.

Influence of Ether Extract of Feces on Prevention of Rickets in Chicks.

	Weight.	Age.	Ash in tibia.
	gm.	*wks.*	*per cent*
Basal ration (average of 6 chicks)..	150	6	32.4
" " plus ether extract of feces from Cow 1 (average of 2 chicks)........................	205	6	44.27
Basal ration plus ether extract of feces from Cow 2 (average of 3 chicks)........................	220	6	43.60
Basal ration plus ether extract of feces from Cow 3 (average of 3 chicks)........................	162	6	35.27

Consequently we decided to determine if the feces of these animals still contained vitamin D.

While such an experiment would not indicate definitely that some vitamin D had not been absorbed yet it would give us an indication as to how completely the vitamin was removed from the tract. For this determination part of the dried feces from each animal was extracted with ether for 40 hours, and this extract evaporated upon a basal rachitic ration for chicks, consisting of 97 parts of yellow corn, 2 parts of calcium carbonate, and 1 part of common salt. The ether was removed from the ration by means of a fan.

Four groups of six chicks each were used, and the ration mentioned above was always fed with skimmed milk *ad libitum*. One group was fed the basal ration, while the other three groups

received the addition of the ether extract of the dried feces of Cows 1, 2, and 3, respectively, at a level equivalent to 5 per cent of the mash as cod liver oil. This level was based upon the assumption that all of the cod liver oil ingested daily remained in the feces.

At the end of 6 weeks, the chicks were killed, the silver nitrate test applied to the proximal end of the tibia, and ash analyses run on the tibias after the usual drying and extraction with alcohol. Some trouble was encountered by the early death of some of the chicks fed the ether extract of the feces. However, a sufficient number of animals was carried through with adequate consumption of the ration to give satisfactory results.

As shown in Table III the chicks in Groups 2 and 3 receiving the extract of the feces from the animals receiving cod liver oil showed an ash content of the tibias of 43 to 45 per cent, which is practically normal, while the ash content of the tibias of the control group was approximately 33 per cent. In Group 4, where no cod liver oil had been fed the cow, the ash of the tibias was approximately 35 per cent, showing the presence of little vitamin D in the ether extract of the feces.

These results indicate that at the level fed there had been very poor absorption of vitamin D from the tract.

SUMMARY.

1. Feeding ½ pound daily of cod liver oil, potent in vitamin D, showed no favorable influence upon the calcium assimilation of liberally milking cows.

2. Vitamin D in the cod liver oil was poorly, if at all, absorbed from the intestinal tract.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

XII. A STUDY OF THE INFLUENCE OF HAYS CURED WITH VARYING EXPOSURE TO SUNLIGHT ON THE CALCIUM METABOLISM OF MILKING COWS.*

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Our previous demonstration of the failure of ultra-violet light (1) applied directly to the cow and of cod liver oil (2) ingested with the ration of the animal to influence favorably the calcium and phosphorus assimilation of milking cows, has turned our attention to a study of the best means of supplying vitamin D to this species. Invariably liberally milking cows are in a negative lime balance during the early part of their lactation period, although they may be fed a most liberal calcium-containing roughage, such as alfalfa hay. It is altogether possible that it is a normal physiological state for a heavy milking cow to be in negative lime balance during the early part of the lactation period, but until this condition has been prevented, if possible, by proper feeding, it will not be known what influence such negative balances have upon the progressive diminution in milk production with advancing lactation. It is for this reason that we are devoting more study to this problem.

In 1925 (3) following the work of Steenbock in this laboratory and of Hess of New York, which showed that antirachitic properties could be imparted to food by direct exposure to ultra-violet

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light, it was demonstrated that the antirachitic potency of hay is dependent upon its exposure to sunlight. Clover hay made with exposure to sunlight showed considerable calcifying power, while clover hay made in the dark was inactive.

For the reasons outlined in the paragraphs above alfalfa hays were cured with varying exposures to sunlight, and their effect upon the calcium metabolism of heavy milking cows was studied. The quantitative antirachitic properties were also determined through experiments with rats. Because of the common importation into this state (Wisconsin) of western alfalfa hays, it was planned to compare hays grown and cured in Colorado with hays grown and cured at Madison, Wisconsin. It was thought that perhaps hays cured in Colorado with its higher altitude and abundance of sunlight would have antirachitic properties superior to hays cured in Wisconsin where the altitude is lower and where the sunshine is generally less abundant. To carry out this plan we secured the cooperation of the Denver Alfalfa Milling and Products Company at Lamar, Colorado; this company kindly consented to cure two alfalfa hays (first cutting) in such a way that the first, Colorado Hay 1, would have little exposure to sunlight, while Colorado Hay 2, cut from the same field and at the same time, would have a very considerable exposure to sunlight. The details of preparing the different hays used in this work were as follows:

Colorado Hay 1.—The alfalfa hay was the first cutting and was removed from the field as soon as it could be handled. It had been cut on one day and allowed to dry in the swath until the next afternoon, when it was raked into windrows. The next afternoon it was bunched, loaded onto a wagon, and hauled to the mill. It was too green and tough to grind. The hay was allowed to dry and cure on the wagon for a few days and just before grinding was spread out on the ground to dry more thoroughly. This procedure involved an exposure to about 3 hours of sunshine after the hay had been gathered from the field.

Colorado Hay 2.—This hay was cut at the same time as Colorado Hay 1. It was allowed to lie in the swath for several days (3 days) and then raked into the windrows to be exposed to the air and sun for several days longer. When in proper condition for grinding, it was loaded onto wagons, taken to the mill, and ground.

From a survey of the methods of preparing the two hays, one is convinced that Colorado Hay 2 was exposed to sunshine very much longer than Colorado Hay 1, although it is apparent that Colorado Hay 1 was also exposed to the sun for some time; the difference between the two hays was a matter of the number of hours of exposure of each. It was altogether possible that Colorado Hay 1 was exposed to the sunshine a sufficiently long period to acquire as much antirachitic potency as Colorado Hay 2.

Wisconsin Hay 1.—About the time these hays were being prepared in Colorado, alfalfa (first cutting) was prepared at our local station. It was cut in the late morning of June 27, 1927. The weather was clear. It was allowed to lie in the swath and was turned with forks on June 28, the weather being clear and sunny. It lay in the swath in the sunshine until 3 p.m., June 28. Consequently it had a fine exposure to sunlight during June 27 and 28. On the evening of June 28, it was bunched and on the morning of June 29 again spread out. The day was clear and sunny. In the afternoon of June 29 it was loaded onto wagons and taken to the barn. Consequently this hay had a curing exposure to sunlight for 3 days. It was ideally cured with no exposure to rain.

These three hays constituted the basis for our metabolism work with dairy cows, and were also the basis for the study of their quantitative antirachitic potency with rats.

In addition to the three hays mentioned above, which were used only for the cow metabolism work, there was a fourth alfalfa hay cured (second cutting) for comparison with the first cutting in antirachitic properties, particularly in the studies with the rat. This hay was also cured at the local station and will be designated Wisconsin Hay 2. It was cut on August 4, the weather being clear and sunny. It was allowed to lie August 4 in the swath in the sunshine. On August 5 it was turned and allowed to lie until 4 p.m. when it was bunched, thus having a fine exposure to sunshine for 2 days. It remained in the cock over the night of August 5. On the morning of August 6 it was spread out and exposed to the clear sunshine until the afternoon of August 6, when it was loaded onto the wagons and taken to the barn. It was ideally cured and sunned for 3 days without exposure to rain.

For the metabolism work three pure bred Holstein cows were selected. They were brought to the station in February, 1928;

Cow 1 freshened on February 18, 1928, Cow 2 freshened February 20, 1928, and Cow 3 freshened February 26, 1928. It was planned to place them in the metabolism work when they were about 6 weeks along in their lactation period. In fact, the metabolism experiment was started on April 3. 2 weeks previous to being placed in the metabolism stalls, all of the cows were given their experimental ration, which consisted of 11 pounds each of Colorado Hay 1 (alfalfa), 25 pounds of corn silage, and 14 pounds of a grain mixture consisting of 35 parts of yellow corn, 30 parts of ground oats, 30 parts of wheat bran, 5 parts of oil meal, and 1 part of common salt. Colorado Hay 1 was selected for the first period of 3 weeks because it was believed that it would show the least antirachitic potency. The experiment was divided into two periods. In the first period of 3 weeks all of the cows received the Colorado Hay 1; in the second period which lasted 4 weeks, Cow 1 received Colorado Hay 2, while Cows 2 and 3 received Wisconsin Hay 1. It was planned to keep the calcium intake alike in both periods. The analyses showed that the hays varied somewhat in the calcium content. To secure a like ingestion of calcium in both periods, it was found necessary to feed during the first period 11 pounds of Colorado Hay 1 daily; in the second period 8.7 pounds daily of Colorado Hay 2 for Cow 1, and 11.2 pounds of Wisconsin Hay 1 for Cows 2 and 3. This made the calcium intake in both periods practically alike for all animals.

There was quantitative collection of the excreta during the 7 weeks period of the metabolism experiment, and weekly composite sampling of the milk for its analysis. None of the animals was bred during the period of the experiment; their consumption was complete and they were quiet and never appeared excited. There is no reason to believe that at any time there was a nervous condition that would influence the normal physiological behavior of these animals. The animals maintained their weights fairly well throughout the experiment, Cow 1 having an initial weight of 1200 pounds on April 2 and a final weight of 1124 on May 21; Cow 2 had an initial weight of 1340 and a final weight of 1255; Cow 3 had an initial weight of 1355 and a final weight of 1311 Milk production throughout the 7 weeks metabolism experiment was well sustained, although from a study of Table I, giving the complete data on calcium metabolism, it will be seen that the cows

TABLE I.

Record of Calcium Balance and Milk Production.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per wk.	Milk per wk.
			Cow 1. Colorado Hay 1.				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
Apr. 3- 9	803.8	12.0	258.5	1074.3	883.5	−190.8	331.1
" 10–16	668.9	13.6	235.6	918.1	882.8	−35.3	318 4
" 17–23	692.2	13.1	239.7	945.0	882.8	−62.2	307.0
			Colorado Hay 2.				
Apr. 24–30	688.7	11.7	232.2	932.6	878.5	−54 1	302.7
May 1- 7	677.7	9.5	217.3	904.5	878 5	−26.0	295 5
" 8–14	684.7	10.5	230.1	925.3	878 5	−46.8	298 2
" 15–21	671.9	8.2	221.9	902 0	878 5	−23 5	294 5
			Cow 2. Colorado Hay 1.				
Apr. 3- 9	694.7	19 2	282.6	996 5	883.5	−113 0	353 7
" 10–16	665.9	25 2	295.8	986.9	882.7	−104.2	356 0
" 17 23	671.1	21 3	288 4	980.8	882.7	−98.1	351.0
			Wisconsin Hay 1.				
Apr. 24–30	648.3	12.0	287.3	947.6	874.1	−73.5	347 7
May 1- 7	639.2	11.6	262.7	913.5	874.1	−39 4	338.4
" 8–14	641.3	9.4	252.0	902.7	874.1	−28.6	338 3
" 15 21	626.4	8.9	263.9	899.2	874.1	−25.1	339 9
			Cow 3. Colorado Hay 1.				
Apr. 3- 9	835.8	13.5	262.2	1111.5	883.5	−228.0	315 6
" 10–16	764.0	17.2	247.9	1029.1	882.7	−146.4	306.7
" 17–23	751.9	14.2	239.6	1005.7	882.7	−123.0	293.2
			Wisconsin Hay 1.				
Apr. 24–30	694.3	7.3	244.4	946.0	874.1	−71.9	294 2
May 1- 7	635.0	12.9	231.8	879.7	874.1	−5 6	283 6
" 8–14	680.2	9.6	228.0	917.8	874.1	−43.7	277.4
" 15–21	680.4	10.4	226.6	917.4	874.1	−43.3	275.7

were all in negative lime balance throughout the entire length of the experiment.

The records of the metabolism data on the calcium balances with the various hays are given in Table I. On Colorado Hay 1 very distinct *negative* lime balances ensued. This occurred with all of the cows. In the case of Cow 1, when the change was made to Colorado Hay 2, there seemed to be but slight reduction in the losses of lime. The losses of lime during the second 4 weeks were about of the same order as the losses of lime during the last 2 weeks of the feeding of Colorado Hay 1. With Cows 2 and 3 there were negative lime balances on Colorado Hay 1, but there appeared to be some diminution in the calcium losses during the feeding of Wisconsin Hay 1. In no case was a positive balance reached with Wisconsin Hay 1, and the results are of such an indecisive character that the most that can be said would be that the Wisconsin cured hay was at least equal to if not better in its antirachitic potency than the Colorado hay.

This last conclusion is supported by the evidence which was secured on the various hays with rats. In the experiments with rats the preventive type of experiment was used and the hays were fed at levels of 5 per cent as additions to the rachitic rat ration, No. 2965.[1] The hays were thoroughly ball-milled before being added to the rachitic ration and observations made not only on the size of the costochondral junctions and the width of the metaphysis through the use of the silver nitrate test, but also the ash content of the femurs after extraction with alcohol. The records of these data are shown in Table II. It will be seen that 5 per cent of any of these alfalfa hays was somewhat below the amount that would furnish sufficient vitamin D for complete calcification. Colorado Hay 1 seemed to show a somewhat lower power of calcification than Colorado Hay 2, although the difference is certainly not large, and the difference between Colorado Hay 2 and Wisconsin Hay 1 is also of a low order. On the basis of the ash content of the femurs the indications would be that Colorado Hay 2 and Wisconsin Hay 1 were much alike in their antirachitic properties and possibly slightly better than Colorado Hay 1. All of these hays contributed some vitamin D to the ration as evidenced by the very much better calcification as compared with the check group. In Table II are also included the data on the

[1] Steenbock, H., and Black, A., *J. Biol. Chem.*, 64, 263 (1925).

TABLE II.

Comparison of Antirachitic Potency of Alfalfa Hays.

Rat No.	Addition to Ration 2965.	Per cent ash in femurs.							Average.	Width of metaphysis.	Size of costochondral junctions.
1–6	+ 5% Colorado Hay 1 (1st cutting).		♀	♀	♂	♂	♀	♂		Medium rachitic metaphyses.	Slight to medium enlargement.
		Femurs.	44.71	42.62	42.72	41.43	38.78	39.70			
			44.45	42 45	43.05	42.03	38.84	40.13			
		Average.	44.58	42.53	42.88	41.73	38.81	39 91	41.77		
7–12	+ 5% Colorado Hay 2 (1st cutting).		♀	♂	♂	♀	♀	♂		Medium rachitic metaphyses.	Slight enlargement.
		Femurs.	45.01	43.57	40.98	43.30	41.21	48 05			
			45.01	43.67	41.37	43.72	40.92	48.36			
		Average.	45.01	43.62	41.17	43 51	41 06	48 20	43.77		
13–18	+ 5% Wisconsin Hay 1 (1st cutting).		♂	♂	♀	♀	♀	♀		Medium to narrow rachitic metaphyses.	Slight enlargement.
		Femurs.	45.75	44.31	43.15	47.83	42 68	43.98			
			45.79	45.38	43.11	48.28	43.73	44 13			
		Average.	45.77	44.84	43.13	48.05	43.22	44 05	44.84		
19–24	+ 5% Wisconsin Hay 2 (2nd cutting).		♂	♀	♀	♂	♂	♀		Medium to narrow rachitic metaphyses.	Medium enlargement.
		Femurs.	44.05	48.25	45.43	44.35	43.48	47.55			
			44.90	49.22	45.22	46.24	43.83	48 69			
		Average.	44.74	48.73	45.32	45.29	43.60	48.12	45.93		
25–30	No addition.		♂	♀	♂	♂	♀	♀		Wide rachitic metaphyses.	Very enlarged and angular.
		Femurs.	24 69	29.69	25.47	36 04	29.18	28.87			
			27.26	30 89	24.74	34.78	28.18	28.12			
		Average.	25 97	30.29	25.10	35.41	28.68	28 44	28.99		

efficiency of Wisconsin Hay 2, which was a second cutting hay and had an ideal curing in the sunshine for 3 days. This hay in its antirachitic potency as measured with the rat also is equal if not slightly superior to Wisconsin Hay 1.

Considerable investigation has been reported on the calcium-phosphorus ratio and its optimum for the rat; little attention has been given this subject in the case of the dairy cow and what influence such a ratio may have upon the optimum utilization of calcium and phosphorus. The ration that we used in the first period of this experiment had a calcium-phosphorus ratio of 1:0.86 with an absolute intake of about 630 gm. of calcium per week and 545 gm. of phosphorus per week, while in the second period for Cow 1 the ratio was 1:0.79. As mentioned above we know very little about the influence of the calcium-phosphorus ratio on utilization of these elements by the cow, but a review of our own experiments in this same field showed that in some experiments where we have had calcium-phosphorus ratios similar to that used in this experiment the lime balance has been positive. For example, in an experiment (4) with a cow giving 40 pounds of milk per day, and with the use of either dry alfalfa or green alfalfa hay, and with the calcium-phosphorus ratio of 1:0.75, the lime balance was positive. In this case the total intake of calcium per week was 560 gm. and the phosphorus intake for the same period 425 gm. Other examples from our records with cows show that where the calcium-phosphorus ratio was 1:0.57 with a calcium intake per week of 950 gm. and a phosphorus intake of 537 gm., the calcium balance was positive. On the other hand other records show that with a calcium-phosphorus ratio of 1:0.57 and alfalfa hay as the roughage, and with a cow milking nearly 50 pounds of milk per day, the lime balance was negative, while the total intake of calcium per week was 650 gm. and the phosphorus 316 gm. Apparently the calcium-phosphorus ratios can be much alike, and yet in some cases the calcium balance positive and in other cases the calcium balance negative. What the effect would be if the calcium-phosphorus ratio was very much widened and the phosphorus made to exceed the calcium, is a question worth consideration. In metabolism experiments with cows (5) on green grasses low in calcium and with the grain portion of the ration rich in phosphorus, the calcium-phosphorus ratio was 1:1.7 and the

calcium balances negative. When these green grasses were fortified with bone meal and the calcium-phosphorus ratio brought to 1:0.86, the balance became positive. How much of this result is to be attributed to a balance of calcium to phosphorus, and how much to actual supply of lime or other factors in the ration, is a question. Where the ratio was 1:1.7 the actual amount of calcium in the ration per week was but 336 gm. Where the ratio was 1:0.86 the supply of calcium per week was 840 gm.

It is evident from the data secured on these hays with the rat that they all possessed antirachitic properties. Colorado Hay 1 was somewhat less potent in this factor than Colorado Hay 2 and Wisconsin Hays 1 and 2. It is also apparent that the Wisconsin cured hays were quite as potent in vitamin D as were the Colorado cured hays. While all of the hays possessed definite antirachitic properties there is no evidence that what they did possess was of sufficient magnitude to control the calcium assimilation of liberally milking cows. Occasionally an alfalfa hay may be found (6) which will maintain calcium equilibrium in cows producing 40 pounds of milk daily, but this situation does not seem to be a common experience in view of the many reports of negative calcium balances observed by various investigators with cows giving above 35 pounds of milk daily and with the use of alfalfa hay. Where the daily milk yield is less than 35 pounds and there is a daily ingestion of 10 pounds of alfalfa of average lime content, calcium equilibrium is likely to be maintained.

While hays may vary considerably in their antirachitic potency due to conditions of curing and sunlight exposure, it is altogether possible that there are factors other than the antirachitic vitamin operating in the absorption of lime from the tract of the cow.

SUMMARY.

1. Alfalfa hays cured in Colorado were no more potent in antirachitic properties than alfalfa hays cured under the best sunning conditions in Wisconsin. All of the hays studied possessed measurable antirachitic properties.

2. However, none of these hays fed in our ration at a level of about 10 pounds per day was able to maintain calcium equilibrium in a liberally milking cow.

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AN ELECTROLYTIC METHOD FOR THE DETERMINATION OF SMALL AMOUNTS OF MERCURY IN BODY FLUIDS AND TISSUES.*

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INTRODUCTION.

Although the therapeutic field of mercury is large and its use in medicine of great importance, in comparison with other therapeutic agents, little is known of its distribution in the body or its rate of elimination. This lack of information is largely due to the inadequacy of methods for the determination of mercury in small amounts. It is not within the scope of this paper to elaborate a complete bibliography of the many methods described in the literature. For such a complete résumé the reader is referred to the paper of Bouton and Duschak (1). We shall discuss only that portion of the literature having a direct bearing on our present problem.

From a careful study of the literature, the problem of mercury determination resolved itself into three problems. First, since physiological fluids present a complex organic structure, an adequate method of digestion was required. This digestion must be sufficient to break existing mercury complexes to a simple inorganic state, without loss of mercury. Secondly, an efficient concentration method was essential, for the concentration of mercury in physiological fluids is necessarily very small. In the third place an accurate method of determination free from complications by interfering agents, and at the same time simple in operation, must be developed.

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HISTORICAL.

In dealing with the determination of mercury in physiological fluids the first problem is that of concentration. For this purpose many methods have been devised. Probably the earliest of these was the precipitation of the metal from solution by employing the electrode potential of a second metal. Jolles (2) in 1895 removed mercury from physiological solutions by the use of zinc dust, the excess of zinc being removed by hydrochloric acid. The mercury was then amalgamated on gold. The mercury has been precipitated on zinc, and solution effected by hydrochloric acid-potassium chlorate mixtures. The mercury was subsequently collected on a Schumacher (3) gold asbestos mat.

François Maurice (4) precipitated the mercury with zinc, dissolving the zinc in HCl and leaving the mercury as a globule.

Eschbaum (5) used copper gauze as the precipitating medium, afterwards distilling off the mercury in a glass tube. Richards and Singer (6) precipitated the mercury from nitric acid solutions with copper foil, claiming complete precipitation in 6 to 7 hours. The last traces were removed by adding fresh copper foil and allowing the solution to stand in contact with it for a further 2 hours. Elliott (7) stated that in this reaction copper dust was preferable to copper gauze. Booth, Schreiber, and Zwick (8) state that in their experience the copper and zinc amalgamation methods do not give quantitative separations. (This finding was also confirmed in this laboratory.) These authors also state that the mercury in physiological fluids exists partially in the colloidal state, and hence would not be precipitated as the metal, on metals above mercury in the electrochemical series

The treatment of the mercury after its deposition is varied. Most authors distill off the mercury in a slow stream of CO_2 and weigh the metal amalgamated on gold. The volatilization of mercury from copper is not usually complete; however, Richards (6) reports total volatilization of mercury from copper in the presence of hydrogen.

In addition to the direct deposition method, other precipitation methods are in general use. Rose (9) obtained mercury in the free state by reduction with stannous chloride, and subsequently weighed it as such. Knovenagel and Ebler (10) precipitated the mercury with hydroxylamine or hydrazine. These precipitations are objectionable since colloidal mercury may readily be formed in the reductions (11–14) particularly if the mercury solution is dilute, as in the case of physiological fluids.

For the final analytical determination of the metal as the free metal or in the ionic state one also has a choice of many methods. By the use of the amalgamation or deposition methods the mercury has been determined directly by the increase in weight of a tube of gold-foil, by titration with iodine, or any similar oxidative reaction, or colorimetrically with diphenylcarbizide.

Volumetrically, mercury has been determined directly in solutions of its salts by precipitation by one of the above methods, and by using iodimetric

titrations of the excess of precipitant. Reductions by arsenious acid, with subsequent titration of the excess of the acid by iodine (15) and potassium permanganate have been carried out quantitatively.

Volhard (16) in his paper on the sulfocyanide determination of the halogens, mentions that mercuric mercury in dilute and neutral solutions may be determined by this reagent. He considered this an interesting side reaction of no great analytical importance. Rupp and Krauss (17) in 1902 showed that mercury can be determined by the sulfocyanide titration if nitric acid is present in considerable amounts. Knox (18) describes this method as one of extreme accuracy if all the mercury is in the high valence form. He finds that in a solution of mercury or mercuric oxide in nitric acid some mercurous salts are always formed. Bouton and Duschak (1) present evidence to show that even minute traces of chlorides vitiate the results by this method. They also state that, following studies of the method of silver determinations, copper, zinc, cadmium, thallium, tin, lead, arsenic, antimony, bismuth, iron, and manganese do not interfere. Since this method presents less interfering agents than the other methods in use, it has been adopted by the authors in the present work.

Probably the most outstanding method of recent origin which provides for a concentration of the mercury, is that of Booth, Schreiber, and Zwick (8). They precipitate the mercury as the sulfide with manganous hydroxide, utilizing the adsorptive power of the latter to carry down by occlusion any mercury in the colloidal form. The decomposition of the sulfide is carried out by the method of Zdrahal (19) with lead chromate. A special tube is used and described by the authors. Magnesite, heated in the closed end of the tube, furnishes a current of gas for the expulsion of the mercury. The mercury was finally determined by drawing it into a calibrated pipette and measuring the length of the column. The accuracy claimed by the authors is high. The method is, however, open to one main criticism: it is laborious and not applicable to general clinical use. It is furthermore, not accurate in the presence of iodides.

Hydrolysis of Mercury Compounds.

Booth, Schreiber, and Zwick (8) report that mercury is present in digests of physiological fluids, in a colloidal form. These colloidal compounds are presumably the oxymercury compounds. An attempt was made by the authors to account for the loss of mercury following ordinary digestion procedures. Three major possibilities presented themselves: (1) that oxymercury compounds, as suggested by Booth, Schreiber, and Zwick (8), may be formed; (2) that the mercury compounds present were volatilized at digestion temperatures; or (3) that reduction of the mercury to the mercurous form may be a necessary preliminary step in the volatilization.

Le Chatelier (20) has shown that the decomposition of mercuric sulfate depends upon the concentration of free acid. With this in mind the authors carried out a series of digestions of mercuric

nitrate in open Kjeldahl flasks, treating them in precisely the same way as one would carry out the digestion for a nitrogen determination.

EXPERIMENTAL.

A known amount of mercuric nitrate in 10 per cent nitric acid was diluted to approximately 250 cc. with water in a Kjeldahl flask. 20 cc. of concentrated sulfuric acid were added and the mixture digested in the usual way. The flask was heated for 20 minutes after the fumes of sulfur trioxide appeared. The contents were then cooled, diluted with an equal volume of distilled water, and titrated with 0.05 N potassium sulfocyanide solution. By this digestion chlorides were found to be absent when the solution was tested with silver nitrate.

Varied amounts of mercuric nitrate ranging from 0.5 to 100 mg. as mercury were used, and complete recoveries were obtained. From this evidence it would appear that under the conditions of the experiment no mercury was lost at the digestion temperature.

Effect of Reduction.

As it was believed at first that these and similar determinations might give satisfactory analytical results, an attempt was made to repeat the work with 250 cc. of urine in place of the water. To this volume of urine known amounts of mercury were added. The results show that some other interfering reaction occurs causing considerable loss of mercury. In ten out of twelve cases this loss of mercury was shown.

Since the acid concentration remained the same in these digestions, it seemed impossible to account for losses of mercury on the basis of hydrolysis. In the preliminary series loss by volatilization had not occurred. This suggested that in the presence of the organic matter of the urine the mercuric compounds were reduced and that these reduced compounds were subsequently lost on heating. Therefore another experiment was carried out in which such reducing agents as starch, sugar, and filter paper were added to the digestion mixture of the first experiment.

In every case where a reducing agent was present a considerable loss of mercury occurred, amounting in some cases to 75 per cent of the added amount.

These results show conclusively that reduction plays an important part in the loss of mercury from solution.

Over 50 urine samples from patients suffering from various pathological disturbances were studied and in the majority of cases low results were obtained. We have been unable to account for the occasional theoretical yield obtained, other than the supposition that the urine was very dilute with respect to organic matter. From these findings any direct titrations of a digested urine was decided to be impossible.

Amalgamation Methods.

From the work of Richards and Singer (6) it appeared that with refined apparatus one might successfully use an amalgamation method for the determination of mercury. After considerable review of the methods for digestion, it was decided to use the acid permanganate digestion of Palme, Lomholt, and Christiansen as described by Booth, Schreiber, and Zwick (8). The digestion mixture was modified as follows:

250 cc. of urine were diluted with 100 cc. of distilled water. To the diluted urine 25 cc. of concentrated nitric acid and 5 cc. of concentrated sulfuric acid were added. About 2 gm. of potassium permanganate were then added to the mixture and 10 cc. of chloroform to prevent foaming. The 800 cc. Kjeldahl flask in which the digestion was carried out was protected by an internal condenser as described by Booth, Schreiber, and Zwick (8). The oxidation was continued, more permanganate being added as required, until a colorless mixture resulted. About 30 minutes were required for this operation. Any excess of permanganate was destroyed by hydrogen peroxide, and the excess of this reagent boiled out. About 1 gm. of finely divided copper was then added and the flask shaken well for 10 minutes, after which it was allowed to stand overnight. The copper was then filtered out, washed with water, alcohol, and ether, placed in a porcelain boat, and ignited in a special apparatus in a current of hydrogen (Fig. 1). The volatilized mercury was absorbed in concentrated nitric acid and titrated with 0.05 N KCNS solution. This apparatus excluded all of the apparent possibilities of loss, noticed in other methods. The results were, however, far from promising, since constantly low results were obtained. Under the conditions of

temperature and the presence of an atmosphere of hydrogen one would expect complete volatilization of the mercury. The only other possibility of loss was in the complete deposition of the mercury on the copper. The profound limitations of the method as a means of concentration were probably due to the fact that the equilibrium between the solution pressure of the mercury deposited on the copper, and the osmotic pressure of the mercury ions in solution, was soon reached. This equilibrium was established at

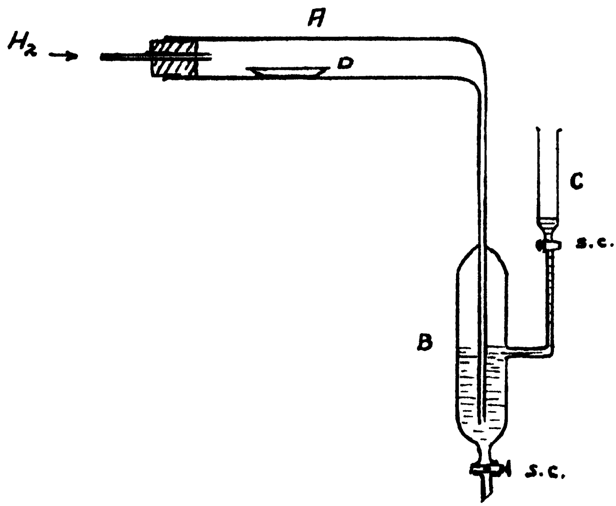

FIG. 1. *A*, combustion tube; *B*, absorption chamber; *C*, filling funnel; *D*, boat containing HgCu; *s.c.*, stop-cock.

a point where a considerable concentration of mercury remained in solution.

Electrolytic Method.

From these studies on the factors affecting mercury determinations we concluded that: (1) hydrolysis could be prevented by the presence of sulfuric acid; (2) heating much above 70° without adequate protection, resulted in serious loss; (3) amalgamation of mercury, or rather the electrochemical deposition on copper, was not complete.

The last of these factors required the development of an elec-

trolytic deposition method. Lomholt and Christiansen (21) used a concentration method consisting in the oxidation by permanganate and sulfuric acid, and precipitation of the mercury as the sulfide, together with copper sulfide. The precipitate was dissolved and deposited on gold electrodes, a voltage of 1.35 to 1.45 volts being used. The electrode was dried and weighed, then ignited and reweighed. The accuracy claimed was 0.03 mg. It was our object from the beginning to develop a method of considerable accuracy involving a minimum expenditure of time, thus avoiding complication of the method. The accuracy claimed by Lombolt and Christiansen would require the use of a micro balance, the operation of which is tedious, and the cost almost prohibitive in the usual laboratory. The low voltage required by these authors was necessitated by the presence of copper which would be deposited with the mercury at higher voltages. The concentration of the sample by precipitation with hydrogen sulfide, and subsequent necessary operations are time-consuming, and usually incomplete. Treadwell (22) states that the great advantage of the electrolytic determination of mercury lies in the fact that good deposits are obtained regardless of the ion to which mercury is attached. Dilute as physiological fluids may be, with carefully controlled voltage and amperage, the mercury should be removed by simple electrolysis of the digested fluid. While electrolytic deposition gave a splendid method of concentration, the authors felt that it had profound limitations as a final method of determination. At the voltage and amperage found necessary for complete deposition (0.5 ampere and 6 volts at 20°) many other metals would also be deposited. Among these are many which would be either normally present in physiological fluids such as magnesium and iron or other metal ions such as arsenic, lead, and bismuth which may be present, due to mixed therapy. Methods of separation of these interfering ions with changes in the normal current density, have been developed. Fredenberg (23) separated arsenic from mercury by electrolyzing with a voltage of 1.7 to 1.8 volts in the presence of nitric acid. Smith (24) separated magnesium by using a current of 0.06 ampere and a pressure of 2 volts. Smith (25) showed that the separation of bismuth from mercury was incomplete. Smith and Moyer (26) show that under an amperage of not greater than 0.5 ampere

lead and manganese are deposited as the oxides on the positive pole. Since manganese in large amounts was present in our digestion mixture, the greatest impurity in the determination was removed.

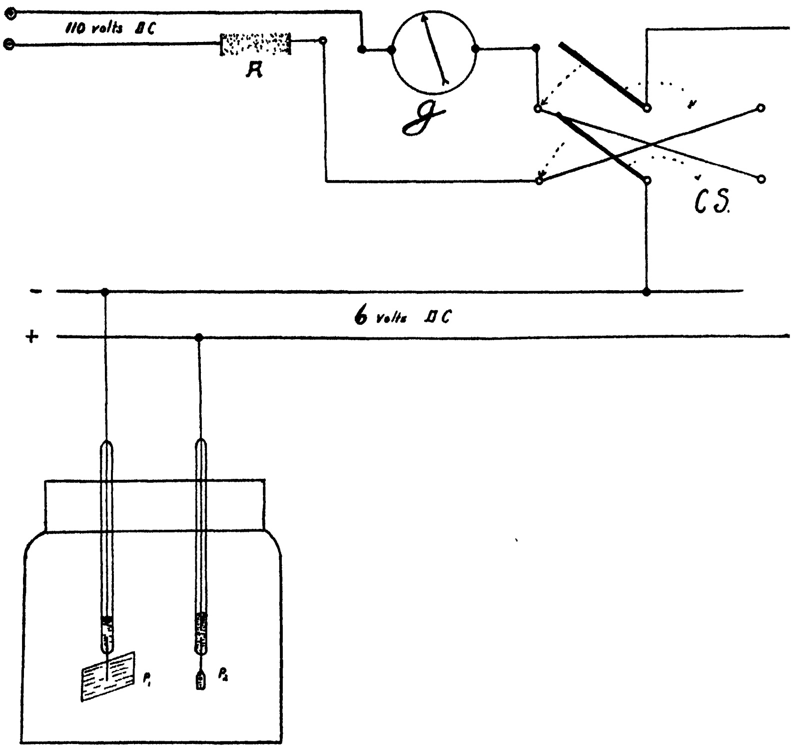

FIG. 2. Diagram of wiring for electrolysis. *R*, carbon resistance; *J*, galvanometer-ammeter; *C.S.*, polarity switch; P_1P_2, electrodes.

It was obvious, from a study of the foregoing literature, that control of the current density for each urine sample to avoid the deposition of interfering agents, was out of the question. As a final method of analysis the electrolytic method did not seem to us to be adequate. It did, however, appear to be the only means of concentration of the samples without loss. It also freed the mercury from interfering anions such as chlorides, bromides, and io-

dides. Since, the mercury could be readily dissolved from the electrode in nitric acid, it was decided to use the electrolytic part of the determination only as a means of concentration. The

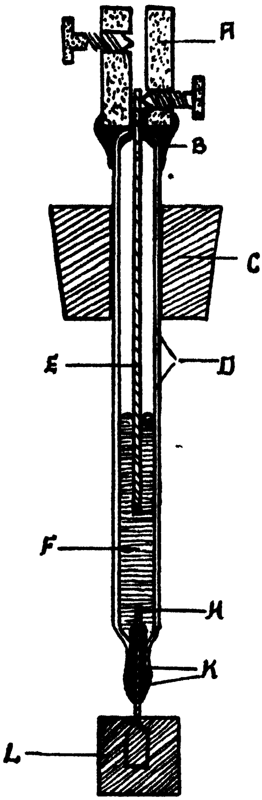

FIG. 3. Platinum electrode for deposition of mercury. *A*, brass binding post; *B*, De Khotinsky seal; *C*, rubber stopper; *D*, glass tube; *E*, copper wire contact; *F*, mercury contact; *H*, platinum contact; *K*, inside lead-glass seal; *L*, platinum electrode.

choice of final methods for determination seemed to be the titration with potassium sulfocyanate. This reagent as shown before is accurate in the presence of all of the interfering agents which might be deposited on the electrode.

Apparatus.

The wiring diagram for the electrolysis is shown in Fig. 2. Ordinary D.C. *house current was reduced by carbon resistance to 6 volts, and the amperage maintained by the addition of electrolyte to the sample.* The electrodes devised (Fig. 3) are easily prepared and cannot spill mercury into the solutions. The platinum foil is about 1 sq. cm. in size, being ample for the amounts of mercury encountered in our work. The set-up used in this laboratory will take care of eight determinations at a time and occupies little space. For electrode vessels, 4 × 4 inch dressing jars were used, which were placed under a wooden rack from which the

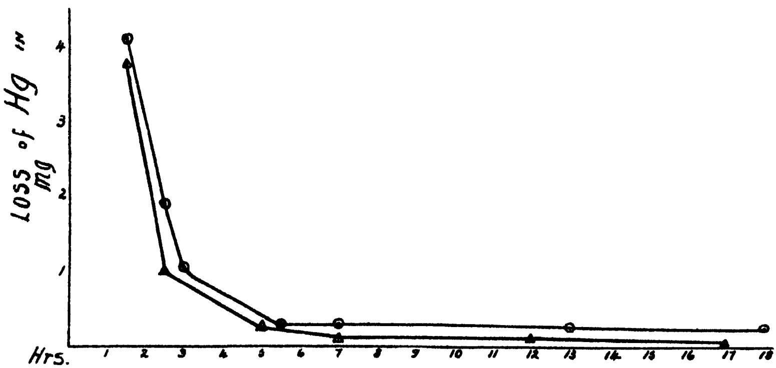

FIG. 4. Deposition of mercury with change of amperage *versus* time. ⊙ = 0.25 ampere; △ = 0.50 ampere. Potential difference = 6 volts. 10 mg sample used.

lower shelf had been removed. The electrodes were then inserted from above, the rubber stoppers fitting the holes in the rack and making the set-up rigid. The entire apparatus can be quickly made from ordinary laboratory supplies.

Effective Electrolytic Conditions.

A study of the effect of voltage and amperage on the deposition of mercury was made and is summarized in Fig. 4. With a potential difference of 6 volts the effective amperage was found to be 0.5 ampere. With this amperage the deposition was complete overnight. Regardless of the amount of mercury taken we have encountered a constant loss of 0.1 to 0.2 mg. of mercury.

Effect of Halogens.

10 per cent solutions of potassium iodide containing 10 mg. of mercury were electrolyzed and complete yields were obtained. In the case of chlorides occasionally a precipitate of mercurous chloride was formed during electrolysis but as the electrolysis proceeds this completely disappeared and theoretical amounts of mercury were obtained.

General Procedure for Urine.

250 cc. of urine were placed in an 800 cc. Kjeldahl flask and diluted with 100 cc. of water. 2 cc. of concentrated sulfuric acid and 25 cc. of nitric acid are added, and the contents thoroughly mixed, the internal condenser being kept in place. 2 gm. of potassium permanganate are added together with 10 cc. of chloroform. (The latter reagent is used to prevent foaming.) Digestion is continued with the condenser in place until odor and color disappear. More permanganate may be added from time to time as required. When digestion is complete transfer the digest to the electrolyzing vessel and allow the current to run overnight. The acid used will give an amperage of approximately 0.5 ampere. If the ammeter shows a lower amperage than this, it is adjusted by addition of nitric acid drop by drop to the electrode vessel. A higher concentration of acid than that given above should be avoided. After the deposition of the mercury the electrode is washed free from electrolyzing solution with distilled water. The deposit is then dissolved in hot fuming nitric acid contained in the titrating vessel. Wash the electrode carefully with distilled water, adding the washings to the nitric acid solution of the mercury. 5 cc. of nitric acid are used in this procedure, and the final volume is made up to 100 cc. with distilled water. 1 drop of potassium permanganate is added to oxidize any mercurous compounds formed from the solution of the mercury in nitric acid. Cool the contents, discharge the permanganate color by adding (drop by drop) 3 per cent hydrogen peroxide. 5 cc. of 10 per cent ferric ammonium alum (chloride-free) is added, and the solution titrated with 0.05 or 0.01 N KCNS with a Folin-Wu micro sugar burette. The titration is continued until the first rose tint appears. From the moment the deposited mercury is removed on

the electrode, all reagents, including the water, must be chloride-free. Blanks on the procedure must be run and the titer subtracted from the total titration value. It would be of distinct advantage to use a standard containing a fraction of a drop of the KCNS as a guide in early determinations. This aid may be dispensed with as the eye becomes accustomed to the end-point. The authors found it readily possible to check each other's titrations to 0.01 cc.

The KCNS was standardized against pure redistilled mercury, previously washed in 3 per cent HNO_3, and filtered through perforated paper. The variation in value of our solution in 8 months was indicated by the initial value of 1 cc. = 1 mg. of Hg, taken in September, and the final value 1 cc. = 0.98 mg. of Hg, taken on June 1. The solutions should, however, be checked about once every 2 weeks against a mercury standard prepared by dissolving redistilled mercury in nitric acid and then diluting until the concentration of nitric acid equals 10 per cent. The strength most convenient, both from the standpoint of accuracy in weighing the mercury, and in view of the strength of the titration solution, is 1 cc. = 10 mg. From this stock solution, a standard of any desired strength may be made by dilution.

Spinal Fluid.

No digestion is required in spinal fluids since the mercury present in such a diffusate could not be in a complicated organic form. In fact, we have found in many cases that urine gives the same values on digestion and without digestion. There are, however, so many exceptions in this case that we digest all urine samples.

Tissues and Feces.

We have found 10 gm. of tissue or feces a convenient amount to work with. The tissues are cut into fine pieces and triturated with concentrated H_2SO_4, sand being used to assist in the disintegration. All tissues form a heavy syrupy fluid under this treatment. The disintegrated tissue is then diluted with water, washed into a Kjeldahl flask, and treated by the same procedure as outlined for urine. For feces 10 to 25 gm. of the 24 hours sample or aliquot is taken and treated with concentrated sulfuric acid in the same way as tissue, the trituration being omitted.

For tissues and feces at least an hour is required for digestion, as compared with 20 minutes for urine. Occasionally a refractory urine sample is found which requires a longer period of digestion. The final digest is colorless and slightly opaque. The chloroform greatly facilitates feces digestion by emulsifying the fatty material present.

TABLE I.

Recovery from Aqueous Solution of $HgNO_3$ and Urine by Electrolytic Method.

Amount added.	Time electrolyzed.	Amount recovered.	Loss.	Sample.
mg.	*hrs.*	*mg.*	*mg.*	
10	16	9.95	0.05	250 cc. water + $HgNO_3$.
10	16	9.90	0.10	
10	16	9.88	0.12	
10	16	9.93	0.07	
10	16	10.00	0.00	
10	16	9.95	0.05	
100	17	99.90	0.10	250 cc. urine + $HgNO_3$.
100	16	100.10	+0.10	
100	16	99.05	0.95	
10	16	9.90	0 10	
10	16	9.85	0.15	
10*	16	9.80	0.20	
5	16	4.95	0.05	
5	16	4.89	0.11	
1	24	0.85	0.15	
1	16	0.89	0.11	

* Undigested.

Results.

In Table I we present values for recovery of added amounts of mercury from water and urine solutions. From these figures we have placed the accuracy of the method at 0.10 mg., although in many instances in our routine work with this method the accuracy has a higher limit of 0.05 mg., when both the loss on electrolysis and the blank are considered. The correction factor used in all determinations which is composed of the average loss after 9 hours of electrolysis, and the blank on reagents, amounts to −0.05 mg. This amount is deducted in practice from the amount obtained.

SUMMARY AND CONCLUSIONS.

Data are presented which indicate:

1. Hydrolytic oxy compounds of mercury are not formed in the presence of sulfuric acid.
2. Reduction of the mercury to the low valence form increases its loss in open digestion.
3. Mercury is not precipitated completely on copper, even after digestion and prolonged standing.
4. An electrolytic method for determining mercury is described, and data showing its accuracy are presented.

In conclusion it may be stated that this method gives a fairly rapid and accurate way of determining mercury in physiological fluids. The upper limit of accuracy is within 1 per cent of the true value, which compares favorably with other biological methods. The method has been used for 400 determinations on physiological fluids without complications, and by these determinations it has been possible to make complete studies of the excretion and distribution of mercury on patients and animals. The combination of the electrolytic with a titration method, obviates many of the difficulties encountered with other methods, owing to the presence of interfering agents, and the necessity of expensive apparatus.

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CHANGES IN THE COMPOSITION OF URINE BROUGHT ABOUT BY SLEEP AND OTHER FACTORS.

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INTRODUCTORY.

The excretion of phosphates is of current interest from many points of view, including its relation to the effect of sleep, the effect of muscular exercise, and the excretion of chlorides. The present report is concerned chiefly with the effect of sleep but bears, too, on the other factors mentioned. It shows, further, that the excretion of phosphates is markedly affected by posture.

Many workers have reported that the excretion of phosphates during sleep at night[1] is greater than during the waking hours of the day (1). Experiments which show the opposite effect—a decrease during sleep—are not lacking, however (2). The most recent work has been practically unanimous in showing a higher excretion of phosphates during sleep than during the day[2] (4, 5). This is contrary to what would be expected if the difference is determined by differences in muscular activity or in the general level of metabolism.

* Dr. Simpson's death from a cardiac disorder of long standing occurred on December 23, 1927. His colleagues will not forget the courage with which, during his last days, he pursued the work to which he was devoted. He was in considerable distress during the time when some of the experiments reported in the next paper were carried out, a few days before his death, but he continued to work vigorously and critically. He prepared the manuscript for this paper practically as it appears. In the following paper I have presented data which he obtained and have endeavored to discuss them briefly in accordance with his ideas.—D. Wright Wilson.

[1] "Sleep" in this paper refers to sleep at night, unless otherwise especially noted.

[2] Results which indicate no change, or are inconclusive, have also been reported (3).

In experiments concerned with the general question of the diurnal rhythm in urine excretion the writer also has found a greater excretion of phosphates during sleep than during the waking hours. However, in some experiments reported below, the changes were reversed. Since this finding was contrary to the more usual one, it was necessary to find some explanation for the discrepancy. It appears that factors other than sleep (or rest) as compared to the waking state (or moderate activity) enter into the results. These factors are (1) the effect of meals, and (2) the effect of change in posture.

EXPERIMENTAL.

The experiments were carried out in much the same way as those previously reported (6). After a night of sleep in the laboratory the subjects were awakened in the morning and given 200 cc. of water. This amount of water was ingested hourly thereafter, and urine was collected every 1 or 2 hours for 24 or 48 hours, part of which time was spent in sleep. The subjects remained in bed and received no food for the duration of the experiments.

The analyses were carried out by well known procedures, for the most part previously enumerated (6). Organic acids were determined in the 48 hour experiment by the method of Van Slyke and Palmer (7). Sulfates were determined in a few of the 24 hour experiments. The usual gravimetric procedure was followed whenever the quantity of urine was sufficient; otherwise the benzidine method of Fiske (8) was used. Phosphates were usually determined by Briggs' (9) modification of the Bell-Doisy procedure. Some of the results reported in Tables VI and VII were obtained with the new method of Fiske and Subbarow (10). These authors studied the Briggs-Bell-Doisy method carefully. The discrepancies which they found when the unknown reading differed greatly from the standard had been recognized by the writer. To avoid error from this source, the quantity of urine was so regulated on the basis of preliminary comparisons that in the final determination, with the standard at 20, the unknown reading was always greater than 18 and less than 22. In over thirty of the first analyses the unknown was also checked by adding to 0.2 mg. of P (as phosphate) a quantity of urine calculated to contain an amount of phosphate sufficient to bring the

TABLE I.

Changes in Composition of Short Interval Urines during the 1st Day of Inanition.

Values are expressed as mM per hour unless stated otherwise.

Time.	Subject MBH-1. May 20, 1923, 100 cc. H_2O hourly.			Subject McL. June 7, 1923, 200 cc. H_2O hourly.			Subject Pf. Oct. 26, 1923, 200 cc. H_2O hourly.		
	Volume.	Phosphate.	Chloride.	Volume.	Phosphate.	Chloride	Volume.	Phosphate.	Sulphate.
	cc.			cc.			cc.		
10 a.m.*	60	0.61	13.9	65	0.96	3.37	240	0.33	0.58
12 m.	277	1.00	27.3	240	1.42	4.17	230	0.45	0.42
2 p.m.	304S	0.97	27.8	200S	1.17	4.36	210	0.78	0.45
4 "	516	1.20	32.0	190	1.90	4.61	250	1.05	0.36
6 "	280	0.97	28.5	240	1.90	4.96	280	1.06	0.32
8 "	425	1.24	40.7	160	1.55	4.80	190	1.08	0.33
10 "	210S	0.29	25.3	150S	1.45	2.85	90	0.76	0.28
12 "	105S	0.80	12.4	180S	1.35	1.69	190S	1.05	0.31
2 a.m	116S	0.89	7.6	140S	1.26	1.66	80S	0.75	0.22
4 "				170S†	1.06	1.86	200S	0.69	0.28
6 "				135	1.03	2.20	265S	0.58	0.33
8 "				235	1.16	2.77	175S	0.52	0.35
10 "				225	1.61	3.22			

Time.	Subject Mo. Dec. 9, 1923, 200 cc H_2O hourly.					Subject Hend. Feb. 8, 1924, 200 cc. H_2O hourly.	
	Volume	pH	Phosphate	Sulfate.	Chloride.	Volume	Phosphate.
	cc					cc.	
10 a.m.*	126	5.8	0.34	0.94	7.5	200	0.56
12 m.	350	6.5	0.22	0.59	5.6	350	0.98
2 p.m.	325	6.6	0.86	0.76	3.0	210	0.95
4 "	230	6.7	1.46	0.49	3.2	150	0.87
6 "	210	6.6	1.30	0.45	2.7	229	0.90
8 "	280	6.5	1.27	0.46	2.8	200	0.80
10 "	160	6.9	1.20	0.42	2.4	110	0.61
12 "	95	6.2	0.93	0.36		110S	0.65
2 a.m.	155	6.0	0.76	0.32	1.4	215A	0.80
4 "	150S	5.9	0.68	0.35	1.2	260S	0.63
6 "	140S	5.9	0.52	0.40	0.9	135S	
8 "	192S	5.0	0.80	0.53	1.7	182S	0.49

S represents sleeping; A, awake.

* 2 hour samples; the 10 a.m. sample represents 8 to 10 a.m. urine.

† Awake during 1st hour.

reading within the required limits. The amount of phosphorus found agreed with the quantity taken with an error of less than 1 per cent. After the general trend of the curves had been more or less established by these analyses, the final determinations were not usually made in duplicate, though the preliminary determina-

TABLE II.

Changes in Composition of Short Interval Urines during the 2nd Day of Inanition.

Subject NBH.

Values are expressed as mM per hour unless stated otherwise.

Time.	Volume.	Chloride.	Phosphate.	Titratable acid.	Ammonia.	Titratable acid + ammonia.	Organic acid.	Urea.	pH	Remarks.
	cc.									
6 a.m.*	76	3.1	1.22	1.25	1.44	2.69	0.52	11.8	5.8	Asleep.
8 "	136	3.2	1.17	1.61			0.68	14.7	5.6	"
10 "	217	3.5	1.13	2.04	2.15	4.19	0.48	16.7	5.3	"
12 m.	130	3.9	1.28	2.04				17.8	5.2	Awake.
2 p.m.	260	4.2	1.62	2.63	2.82	5.45	1.11	20.1	5.2	"
4 "	300	5.2	1.70	2.65	1.68	4.33		18.5	5.3	"
6 "	370	6.2	2.03	2.81	3.16	5.97	1.14	19.4	5.4	"
8 "	266	7.4	2.23	2.90	3.30	6.20	0.70	19.6	5.3	"
10 "	200	9.3	1.94	2.52	3.48	6.00		18.2	5.3	"
12 "	145	5.9	1.45	1.70	3.51	5.21		15.5	5.4	Asleep.
2 a.m.	171		1.41	1.48	3.14	4.62		15.4	5.5	"
4 "	200	3.7	1.26	1.60	3.39	4.99	0.86	15.4	5.5	"
6 "	133	2.9	0.98	1.50				16.0	5.3	"
8 "	226	3.4	0.98	1.52	4.48	6.00	0.63	15.0	5.3	"
1st day.	310	17.1	2.27	2.08	2.71	4.79		25.8	6.6–5.6	
2nd "	455	10.5	3.12	4.26	6.72	10.98		34.7	5.5–5.2	

* 2 hour samples; in this case sample represents 4 to 6 a.m. urine.

tions (for establishing the final dilution) showed general agreement with the final determinations.

The excretion of phosphates during the 1st day of inanition is shown in Table I, together with chlorides and sulfates in some instances. The results obtained during the second 24 hours of the 48 hour experiments are shown in Tables II and III. 4 hour

urine samples were collected during the first 24 hours of the 48 hour experiments to establish the general level of excretion of the various urinary constituents and no points of difference from the previously published results were noted.

TABLE III.

Changes in Composition of Short Interval Urines during the 2nd Day of Inanition.

Subject GCP.

Values are expressed as mM per hour unless stated otherwise.

Time.	Volume.	Chloride.	Phosphate.	Titratable acid.	Ammonia.	Titratable acid + ammonia.	Organic acid.	Urea.	pH	Remarks.
	cc.									
6 a.m.*	145	3.58	0.86	1.19	2.31	3.50	1.7	16.0	5.6	Asleep.
8 "	229	3.50	1.08	1.52	2.48	4.00	2.3	19.2	5.6	"
10 "	287	5.30	1.19	1.86	2.59	4.45	3.4	23.4	5.5	"
12 m.	295	6.80	1.55	2.23	2.52	4.75	3.6	23.5	5.5	Awake.
2 p.m.	285	7.35	2.06	2.52	2.44	4.96	3.3	22.2	5.5	"
4 "	182	6.20	2.08	2.56	2.44	3.72	3.1	20.8	5.4	"
6 "	250	6.08	2.07	2.57	2.35	4.92		21.0	5.6	"
8 "	154	5.94	1.99	2.40	2.59	4.99	3.5	21.0	5.5	"
10 "	161	3.97	1.76	1.96	2.40	4.36	2.6	19.1	5.7	"
12 "	183	1.94	1.58	1.72	2.61	4.33	2.3	18.3	5.8	Asleep.
2 a.m.	179	1.69	1.54	1.68	3.02	4.70	2.2	17.1	5.7	"
4 "	156	2.20	1.34	1.52	2.80	4.32	2.2	18.2	5.6	"
6 "	205	2.34	1.26	1.50	3.12	4.62	2.3	19.4	5.6	"
8 "	142	2.00	1.29	1.80	3.56	4.36	3.3	20.6	5.4	"
1st day.	317	9.3	1.8	2.0	4.3	6.3	3.4	26.2	7.1–5.5	
2nd "	494	8.0	3.3	4.0	5.5	9.5	5.6	39.9	5.8–5.4	

* 2 hour samples; the 6 a.m. sample represents 4 to 6 a.m. urine.

DISCUSSION.

Phosphate excretion is lower during sleep at night than during the day. The decrease on falling asleep is small compared to the decrease in chloride excretion which occurs at this time. The increase in phosphate excretion after waking in the morning of the first day of inanition is simultaneous with the increase in chloride excretion in some experiments (Subjects MBH-1, McL,

Table I). It is the opinion of the writer that this is the result which would always be obtained if the excretion of chlorides was not affected by a "washing out" process. This process is effective at the beginning of the experiments and is associated with the beginning of the ingestion of the large amounts of water.

With Subject Mo this washing out effect is seen during the 1st hour after waking, when urinary pH and phosphate excretion are still low, and continues, in the case of chlorides, through the 2nd hour after waking. With Subject Pf, this effect is seen in the case of sulfates during the 1st hour after waking.

During the second 24 hours of such experiments when the beginning of water ingestion is not a factor, the increase in phosphate excretion on waking in the morning and the decrease on falling asleep at night are simultaneous with the more marked changes in chloride excretion in the same direction (Tables II and III). On both days phosphate excretion clearly reaches a maximum during the afternoon, as shown by Fiske (11).

The experiments reported in Tables I to III were completed 5 years ago. On account of their lack of agreement with the results of others in respect to phosphate excretion the validity of the writer's experimental procedure for experiments concerned with the effect of sleep came into question. The onus of explaining the discrepancy rested with the writer since other workers had not, as he, interrupted the sleep of their subjects. A study of chloride excretion had indicated that the procedure used was valid, since marked changes in this urinary constituent occurred at exactly the time the subjects fell asleep or wakened from sleep (12).

In comparing the writer's experiments with those of others, two differences besides the continuity of sleep were noted: (*a*) the subjects of the present experiments remained in bed, while in the case of other workers the subjects were more or less active during the day, and further (*b*) the writer's subjects received no food, while the subjects of the experiments of others were fed. Accordingly, experiments were undertaken to see if either of these factors would account for the discrepancy.

In the experiments reported in Table IV, the procedure was the same as in the previous ones except that two meals were fed.[3]

[3] In these experiments the subjects spent the night preceding the experiment in a dormitory near the laboratory. They had breakfast before coming to the laboratory.

These were not free from phosphates, since it is not indicated that any care was taken in this respect in the experiments which these were designed to check.

TABLE IV.

*Phosphate Excretion by Subjects Remaining in Bed, but Given Food.**

Time.	Subject PJK. Volume.	Subject PJK. Phosphate.	Subject PJK. Remarks.	Subject BD. Volume.	Subject BD. Phosphate.	Subject BD. Remarks.
1926	*cc.*	*mM*		*cc.*	*mM*	
Feb. 20						
11 a.m.	86	39.8	To laboratory, 10 a.m.			To laboratory, 10 a.m.
12 m.	227	41.4		425	16.1	
1 p.m.	260	41.8	Meal, 12.15.	200	17.9	Meal, 12.15.
2 "	110	25.4		250	18.7	
3 "	420	84.8	Defecated.	60	17.0	
4 "	360	92.6		60	32.1	
5 "	325	73.6		270	27.9	
6 "	400	81.2	Meal, 5.30.	295	34.1	Meal, 5.30.
7 "	305	78.6		205	32.7	
8 "	175	116.2	Sleeping.	130	39.2	Sleeping.
9 "	180	104.0	"	205	39.0	"
10 "	115	65.5	"	275	39.3	"
11 "	290	142.2	"	225	41.7	"
12 "	300	103.6	" complained of heat.	160	43.3	"
1 a.m.	530	104.0	Sleeping.	145	44.4	"
2 "	320	70.1	"	170	37.9	"
3 "	350	48.3	" lightly.	225	36.2	"
4 "	270	39.1	"	270	34.2	"

* Subjects drank 200 cc. of water hourly throughout the experiment. Meals consisted of veal loaf sandwiches with lettuce and mayonnaise, two boiled eggs, salt, large dish of ice cream, glass of milk, one cup of coffee at noon.

Phosphate excretion is now higher during sleep than during the waking hours.

Both meals were followed by an increase in phosphate excretion. The effect in each instance was delayed for 2 hours in the case of Subject PJK. It was delayed for still another hour in the case of Subject BD, possibly on account of a nap during the 3rd

hour. Phosphate excretion reached a higher level after the second meal than after the first in both instances. The effect of the second meal was probably superimposed on that of the first. In neither case was high phosphate excretion necessarily associated with large urine volumes. The markedly higher phosphate excretion of Subject PJK is doubtless due to the larger amounts of milk which this subject ingested on the day preceding the experiment.

The effect of the second factor in which the experiments reported by the writer differed from those of other workers—the effect of activity during the day—is shown in Table V. The subjects of these experiments spent the day in the laboratory, reading in a chair, studying at the microscope, or working at a laboratory bench. They received no food. Phosphate excretion is again greater during the night than during the day.

These two sets of experiments explain the discrepancy between the writer's results and those of other investigators.

The higher level of phosphate excretion during the day shown in Tables I to III may be ascribed either to the effects of muscular exercise *per se* or to the effect of sleep on metabolism in general (in which muscle metabolism obviously plays an important part). However, the high phosphate excretion during sleep found in experiments of others and in the experiments of the writer, reported in Table V, cannot be explained thus. Here the subjects were active during the day. If activity were the determining factor, the level of phosphate excretion during the night should be low. It would also be expected that the difference in this direction would be greater than in experiments (Tables I to III) where the only difference in activity was that between sitting up in bed during the day and lying in bed asleep in the night. On the contrary, not only does the degree of difference fail to accord with the expectation, but the difference is in the opposite direction. Some factor more potent than either a difference in metabolism in general or in muscle metabolism in particular must have determined the results in these experiments.

In this connection it was of interest to note that Kleitman (5) had found (contrary to the more usual result in recent work) a depression in phosphate excretion during periods of exercise and to

TABLE V.

*Phosphate Excretion by Subjects Not Remaining in Bed and Not Given Food.**

Time.	Subject LL.			Subject TL.			Subject SL.		
	Volume.	Phosphate.	Remarks.	Volume.	Phosphate.	Remarks.	Volume.	Phosphate.	Remarks.
1926	*cc.*	*mM*		*cc*	*mM*		*cc.*	*mM*	
Jan. 30–31									
11 a.m.	55	0.61	At work in laboratory.	90	0.79	To laboratory at 10 a.m.	60	0.39	To laboratory at 10 a.m.
12 m.	213	0.75	" "	70	0.90	Reading and walking.	205	0.43	Reading and walking.
1 p.m.	84	0.70	Working and reading.	80	0.81	At microscope.	30	0.68	At microscope.
2 "	355	0.66	Walking slowly.	260	0.81	" "	300	0.75	" "
3 "	175	0.73	Reading.	150	1.00	" "	340	0.83	" "
4 "	340	0.56	"	175	0.95	" "	165	0.95	" "
5 "	210	0.44	"	85	0.94	Walking.	140	1.26	Walking.
6 "	235	0.46	"	270	0.94	"	230	1.25	"
7 "	233	0.34	"	275	0.76	Reading.	175	1.27	Reading.
8 "	235	0.56	"	225	0.95	"	215	1.26	"
9 "	210	0.46	In bed.	195	1.12	In bed, asleep.	465	1.44	In bed, asleep.
10 "	57	0.41	Asleep part time.	185	1.19	Asleep.	130	1.64	Sleeping.
11 "	180	0.43	"	240	1.40	"	100	1.57	"
12 "			"	105	1.50	"	190	1.60	"
1 a.m.	97	0.58	"	180	1.56	"	120	1.46	"
2 "	225	0.77	"	215	1.51	"	135	1.22	"
3 "			"	200	1.24	Sleeping restlessly.	240	1.33	Sleeping restlessly.
4 "			"	250	1.14	" "	170	1.12	" "
8.30 a.m.	305	0.60	"						

* Subjects received 200 cc. of water hourly.

TABLE VI.

Effect of Lying in Bed (Prone) and of Standing Up, on Phosphate Excretion.

Time	Volume	Phosphate.		Chloride		Activity.
		Subject Gr.				
1926	*cc per hr.*	*mM per hr.*	*mM per 100 cc.*	*mM per hr.*	*mM per 100 cc*	
Apr. 25–30						
8–9.30 a m.	40	0.95	2.37	5.92	14.8	In bed, prone.
9.30–11 "	23	0.40	1 74	4.39	18.8	Stood up.
11 a m –12.30 p m.	59	0.84	1.42	8.28	13.7	In bed, prone
		Subject Hu.				
8–9 30 a m.	31	1.32	4.21			In bed, prone
9 30–11 "	27	0 91	3.40			Stood up.
11 a m –12.30 p.m	43	0 95	2.18			In bed, prone.
		Subject Vin.				
8–9.30 a m.	27	1.79	6 7			In bed, prone.
9.30–11 "	20	1.01	5.1			Stood up.
11 a m.–12.30 p.m.	28	1.07	3.8			In bed, prone.
		Subject Meg.				
7–9 a m.	191	0.457	0.24	3.68	1.9	In bed, prone
9–11 "	35	0.305	0.87	2.02	5.8	On feet.
11 a m.–1 p.m.	235	0.710	0.30	4.19	1.8	In bed, prone
		Subject Vir.				
7–9 a m.	410	0.486	0.12	8.75	2.1	In bed, prone.
9–11 "	42	0.185	0.44	6.74	15.8	On feet.
11 a.m.–1 p.m.	255	0.546	0.21	10.15	4.2	In bed, prone.
		Subject Van.				
7–9 a.m.	375	0.533	0.14	6.07	1.6	In bed, prone.
9–11 "	108	0.442	0.41	5.81	5.4	On feet.
11 a.m.– 1 p.m.	260	1.122	0.44	7.02	2.7	In bed, prone.

note that the subjects he used were lying down during the resting control periods.[4]

Consideration of these results led to the suspicion that change of posture might have been the more effective factor governing phosphate excretion, not only in Kleitman's experiments on exercise and sleep but also in the experiments of others concerned with the effect of sleep on phosphate excretion, and in the experiments reported by the writer in Table V. Lying down has been shown to cause diuresis and an increase in chloride excretion (13). It seemed possible that the excretion of phosphate might be similarly affected.

The experiments shown in Table VI were designed to test this possibility. The subjects stood on their feet inactive for 1.5 hours between periods of lying prone in bed for the same period. It will be seen that phosphate excretion is markedly decreased during the period of standing. A great decrease in water excretion accompanies this decrease in phosphate excretion. It was impossible to prevent this even when, as in the last three experiments, large amounts of water were ingested before the experimental period proper. The following considerations indicate that the changes in phosphate excretion are something more than secondary to the volume changes. In the first place Wigglesworth and Woodrow (14) have shown that marked changes in urine volume (diuresis) do not affect phosphate excretion. It seems possible, nevertheless, that phosphate may fail to be excreted if urine volume is very small, and that this may be the case in the middle periods of the first three experiments of Table VI. In the last experiment, however, the decrease in phosphate excretion in the standing period is marked although urine volume is large and the possibility of retention remote. In the second place, phosphate excretion is more markedly affected by standing than chloride excretion. If variations in volume were the determining factor, it would be expected that chloride excretion would be affected in greater degree than any other urinary constituent.

The results reported in Table VI resemble those obtained by

[4] In the case of most other investigators it is not possible to tell from the published reports what the posture of the subjects was during the resting periods.

Kleitman and attributed by him to the effect of muscular exercise. The experimental procedure differed from that of Kleitman only in that, during the middle period, the subjects did not walk vigorously, but simply stood around the laboratory. The present results indicate that the low phosphate excretion during exercise in Kleitman's experiments may be due to the differences in posture rather than to differences in muscular activity.[5]

This conclusion is supported by the series of experiments reported in Table VII. Here a period of vigorous walking was included between two periods of standing around the laboratory. These experiments are similar to those of Kleitman, except that the change of posture is eliminated. The definite decrease in phosphate excretion during the period of walking which was found in Kleitman's experiments now fails to appear. The results are not clear cut and are difficult to explain. They are most easily explained as due to an effect of exercise on phosphate excretion which may appear either during the period of exercise, or may be delayed until after this period.

In the first experiment there was a definite increase in phosphate excretion during the period of exercise. There seems also to have been a delayed effect extending into the third (standing) period. In the second and third experiments there is no marked change in phosphate excretion during the period of exercise itself. The high values following the exercise might be due to a delayed effect. In the fourth experiment the first part of the period of exercise is accompanied by a marked increase in phosphate excretion. The writer sees no explanation for the high value for the second standing period following the exercise in this experiment. The fifth experiment might be taken to support Kleitman's view of a low excretion of phosphate during muscular exercise, especially if the low value for the standing period following the exercise is attributed to the comparatively small urine volume. This amount of urine (84 cc. in 1.5 hours) is not so small as to cause phosphate retention, however, while mere variations in volume would not be expected to influence phosphate excretion, as noted above.

It has been shown by Wilson and his coworkers (15) and by Havard and Reay (16) that phosphate excretion is high following

[5] In Kleitman's experiments, too, volume changes are great and tend to confuse the results.

short periods of strenuous exercise. In the experiments of these workers the periods of exercise were too short and the exercise

TABLE VII.

Effect of Period of Walking between Periods of Standing, on Phosphate Excretion.

Time.	Volume.	Phosphate.	Activity.
	Subject CC.		
1926	*cc. per hr*	*mM per hr.*	
8-9.30 a.m.	314	0.35	Standing.
9.30-11 "	307	0.61	Walking.
11 a.m.-12.30 p.m.	107	0.77	Standing.
	Subject BF.		
8-9.30 a.m.	33	0.42	Standing.
9.30-11 "	140	0.44	Walking.
11 a.m.-12.30 p.m.	107	0.61	Standing.
	Subject WDL.		
1-2 p.m.	73	30.3	Standing.
2-3 "	90	31.9	Walking.
3-4 "	90	43.3	Standing.
4-5 "	150	35.3	"
	Subject JDB.		
1-2 p.m.	370	15.1	Standing.
2-2.30 p.m.	755	29.2	Walking.
2.30-3 "	450	20.8	"
3-4 p.m.	765	20.7	Standing.
4-5 "	875	26.7	"
	Subject AC.		
8-9.30 a.m.	267	1.52	Standing.
9.30-11 "	260	1.01	Walking.
11 a.m.-12.30 p.m.	83	0.74	Standing.

itself too strenuous to permit the collection of urine samples during the exercise. It might be expected from their results, however, that, if the exercise had been continued, the delayed

effect would be included in the period of exercise itself. On the other hand, it might be expected that the immediate effect would be small and that the delayed effect would cause confusion by extending into the succeeding control period. It is, therefore, not surprising that the results reported in the present paper are not clear cut. They are presented here in order to indicate why the literature on the effect of muscular exercise on phosphate excretion shows so many contradictory results[6] and to prevent erroneous conclusions being drawn from the experiments of Kleitman. The effect of long periods of mild exercise is studied better by comparing phosphate excretion on days of exercise with phosphate excretion on rest days, as has been done by Embden and Grafe (17) and Campbell and Webster (18), rather than by comparing exercise and rest periods on the same day.[7]

That phosphate excretion, like chloride excretion, is lower during sleep than during the waking hours is of interest in connection with the question of an antagonistic action between phosphate and chloride excretion. Originally it was for the purpose of gaining light on this point that chloride determinations were carried out in the experiments of Table VII whenever considerable variations in the phosphate values appeared. Embden and Grafe (17) in their studies of phosphate excretion during muscular exercise noted that when phosphate excretion was increased chloride excretion was correspondingly decreased, and *vice versa*, and gave this phenomenon the name "antagonism." Röckemann (20) subsequently found this effect when phosphate excretion was increased by the ingestion of sodium dihydrogen phosphate. Eichholtz and Starling (21) in perfusion experiments with heart-lung-kidney preparations have found a mechanism which would explain this antagonistic action. The results of others, showing a high rate of phosphate excretion during sleep, when taken into

[6] It appears from the results reported in the present paper that the effect of posture has determined the results in many experiments. It is impossible to review the literature from this standpoint, as most workers fail to note the posture of their subjects during the normal rest periods.

[7] Recent work by Havard and Reay (19) shows that the phosphate concentration of the blood is likewise affected by posture and minor activity. This work indicates that in a study of the effect of mild exercise on phosphate excretion, activity and posture should be regulated for a considerable period before the experimental period proper.

consideration with the writer's results, which show a low rate of chloride excretion during sleep, might have been taken as another indication of this antagonism. The present experiments demonstrate that, as far as sleep is concerned, there is no essential antagonistic action.

According to the results of previous experiments on phosphate excretion during sleep this urinary constituent occupied a peculiar position which it was necessary to explain. Broadhurst and Leathes (22) postulated an especial relation to nerve metabolism which was assumed to be higher at night than during the day. Campbell and Webster regarded the high phosphate excretion as necessary to provide for the excretion of large amounts of acid associated with the nocturnal acidosis.

The data in Table II show that the curve for phosphate excretion closely parallels the curves for water, titratable acid, and organic acid and, less closely, the urea curve. Thus the effect of sleep on phosphate excretion is in no way unique.

General Remarks on the Constitution of Urine During the 2nd Day of Inanition.

A study of the data in Tables II and III shows that phosphate, organic acid, titratable acidity, and water comprise a group of urinary constituents which shows a maximum level of excretion during the day which is approximately twice the minimum level during the night. A second group of urinary constituents shows a night level about 20 per cent below the day level. This is comprised of urea, sulfates (as indicated by Table I), and possibly uric acid.[8] This effect is sufficiently accounted for by their relation to metabolism according to which the 20 per cent decrease would be expected (Benedict and Carpenter (24)). The constancy of creatinine excretion previously observed likewise needs no especial explanation. Of all urinary constituents, chloride is unique. The day level even on the 2nd day of inanition is 4 to 5 times the night level. A study of the chloride content of the blood during sleep is under way, which it is hoped may bring light on this peculiarity.

[8] The results for uric acid will be found in the first paper (23) of this series. There is a large washing out effect in the case of this urinary constituent.

The experiments in Tables II and III were originally undertaken for purposes other than those previously indicated.

(*a*) It had been found that, in 1 day experiments, comparatively large amounts of all urinary constituents were excreted during the morning. Chloride and water excretion were especially high, and showed a marked decrease during the afternoon or evening. It seemed possible that this effect might have been due, not to any diurnal rhythm, but rather to the large amounts of water ingested combined with an excess of chlorides ready for excretion. Extension of the same regimen through a 2nd day would get around this effect. Chloride excretion is seen to show the same characteristics on the 2nd day as on the 1st.

(*b*) The large urine volumes in the 1st morning were accompanied by high pH values, while a great decrease in urinary pH was simultaneous with the decrease in water output. On the 2nd morning of such experiments, immediately before their termination, the pH values were low. The values approached the lowest found by Henderson and Palmer (25) and would therefore not be expected to show the fall during the 2nd day that characterized the 1st day. Extension of the experiments through the 2nd day would decide whether there was an obligatory relationship between pH and changes in the level of water excretion.

The hope that urinary pH would be constant was fulfilled. The variation in pH was now only 0.4 units. Urinary volumes still showed marked variations, nevertheless, and no relation was seen between these and the small fluctuations in pH. This is in agreement with the conclusion of Hubbard and Munford (26). Chlorides again exhibited the marked variations seen on the 1st day of inanition.

Titratable acidity, ammonia, and organic acids were followed during the 2nd day for the purpose of determining if any acidosis developed which would be so great that its influence would be expected to determine any abnormalities in urine excretion. The levels of excretion of all these constituents were somewhat higher than during the 1st day of inanition, as seen in Table VIII. Results previously published and those for the 1st day in the case of the subjects of the present 2 day experiments showed a higher excretion of titratable acidity, ammonia, and organic acids during the night than during the day. On the 2nd day, except for am-

monia, the reverse is the case. Titratable acid and organic acid are now excreted in greater amount during the day. This is no doubt due to the developing acidosis caused by the greater activity of the day. It is this which determines the higher level of excretion rather than the acidosis of sleep caused by the depression of the sensitivity of the respiratory center.

Cohen and Dodds (27) have published analyses of short interval urines extending over the 1st day of inanition. In their experiments, the subjects were active during the day, did not sleep during the night, and ingested no water.

TABLE VIII.

Changes in Composition of Day and Night Urines during 2 Days of Inanition. Subject JCP.

Values are expressed as mM per hour, unless stated otherwise.

	Volume.	Chloride.	pH	Phosphate.	Titratable acid.	Ammonia.	Titratable acid + ammonia.	Organic acid.	Urea.
	cc.								
1st day.									
10 a.m.–10 p.m., awake.	175	6.8	7.1–5.9	0.80	0.78	1.5	2.28	1.5	11.6
10 p.m.–8 a.m., asleep.	142	2.5	5.8–5.5	0.97	1.23	2.8	4.03	1.9	14.6
2nd day.									
10 a.m.–10 p.m., awake.	221	6.0	5.4–5.7	1.9	2.37	2.5	4.87	3.2	21.2
10 p.m.–8 a.m., asleep.	173	2.0	5.8–5.4	1.4	1.64	3.0	4.64	2.5	18.7
1st day.									
11 a.m.–9 p.m., awake.	216	13.1	6.4–6.6	1.06	0.82	1.35	2.17	0.38	13.4
9 p.m.–7 a.m., asleep.	94	4.0	6.0–5.6	1.21	1.26	1.36	2.62	0.47	12.4
2nd day.									
11 a.m.–9 p.m., awake.	280	6.5	5.2–5.4	1.90	2.70	2.89	5.59	0.98	19.2
9 p.m.-7 a.m., asleep.	175	4.0	5.5–5.3	1.22	1.56	3.83	5.39	0.74	15.5

SUMMARY.

To study the effect of sleep on the phosphate excretion, subjects were kept in bed without food and were given water hourly. Urine was collected every 2 hours. With this procedure the phosphate excretion was found to be lower during sleep (at night)

than during the waking hours. Similar results were obtained on the 1st and 2nd days of fasting. On both days the phosphate excretion reached a maximum in the afternoon, as found by Fiske.

As the decreased excretion of phosphate during sleep is contrary to many of the published observations, studies were made to explain the discrepancy.

When two meals were given during the day, the régime being otherwise unchanged, a higher phosphate excretion was observed during the night.

When no food was eaten but the subjects were allowed to arise and be moderately active during the day, the phosphate excretion was higher during the night.

Posture affects the phosphate excretion. There was a marked decrease in excretion of phosphate while the subjects were standing inactive from that occurring while lying prone. A considerable decrease in water excretion which also occurred is thought not to have caused the decreased phosphate excretion.

On the 2nd day of an experiment without food the excretion of phosphate, organic acid, titratable acidity, chlorides, and water showed characteristic variations, while the pH values remained practically constant.

These experiments emphasize the necessity of carefully controlling many of the factors which enter into the daily routine in order to study the effect of specific procedures on the composition of the urine.

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THE EFFECT OF BREATHING RELATIVELY HIGH CONCENTRATIONS OF CARBON DIOXIDE ON THE URINARY EXCRETION OF WATER.

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In a previous paper (1) experiments were reported which indicated that the urinary excretion of water is decreased when high concentrations of CO_2 are breathed. The experiments were carried out on dogs accustomed to the routine of the experiments. Further studies on untrained dogs showed that other factors, such as fear and excitement, may possibly play such an important rôle that a more thorough study should be made, instituting more rigorous controls. Experiments which seem to satisfy all the needs are reported below. Added evidence is obtained from experiments carried out on human beings where certain psychical factors were probably absent.

EXPERIMENTAL.

The procedures used are described in detail in the previous paper (Simpson and Wells (1)). In brief, the dog was placed on an animal board, and a mask consisting of a glass jar was placed over the nose. Air or gas mixtures high in CO_2 were passed through the mask for certain periods during which the urine was collected and the volume noted. The dog was accustomed to the experimental routine for several days before the experiments reported below were carried out. During these experiments the dog lay quietly with no periods of apparent excitement or struggling.

Table I records the data of three experiments on one dog. It will be noted that in all three experiments breathing CO_2 mixtures through the mask caused a great decrease in the volume of urine

TABLE I.

Effect of Breathing CO_2 on Excretion of Water.

Time.*	Period No.	Collection interval.	Urine volume.	Remarks.
Dog 3. Nov. 25, 1927. 400 cc. water given each half hour from 9–10 a.m., 300 cc. at 10.30 and 11, 200 cc. each half hour thereafter.				
		min.	*cc. per 30 min.*	
11.56 a.m.	1	31	165	Free in cage.
12.25 p.m.	2	29	217	" " "
12.57 "	3	32	75	On board, air through mask.
1.28 "	4	31	135	Free in cage.
1.59 "	5	31	251	On board, air through mask.
2.31 "	6	32	281	Free in cage.
3.02 "	7	31	208	On board, 10 per cent CO_2 in O_2 through mask.
3.34 "	8	32	211	On board, air through mask.
4.04 "	9	30	70	" " 50 per cent CO_2 in O_2 through mask.
Dog. 3. Nov. 26, 1927. 400 cc. water given at 9 and 9.30 a.m., 300 cc. at 10, 200 cc. each half hour thereafter.				
11.04 a.m.	1	37	300	Free in cage.
11.29 "	2	25	132	On board, mask on 5 min.
11.59 "	3	30	115	Free in cage.
12.23 p.m.	4	24	144	On board, air through mask.
12.50 "	5	27	50	" " CO_2† through mask.
1.22 "	6	32	104	" " mask off.
Dog 3. Nov. 29, 1927. 400 cc. water given each half hour from 9–10 a.m., 200 cc. each half hour thereafter.				
11.08 a.m.	1	33	50	Free in cage, excited (?).
11.30 "	2	22	109	On board, air through mask.
12.03 p.m.	3	33	264	Free in cage.
12.35 "	4	32	140	On board, air through mask.
1.07 "	5	32	140	Free in cage.
1.38 "	6	31	39	On board, CO_2‡ through mask.
2.09 "	7	31	82	" " air " "
2.39 "	8	30	160	" " " " "

* The time given represents the time when the period was completed.

† Gas mixture used, 25 per cent CO_2, 25 per cent O_2, 50 per cent N_2. Animal panted a great deal during this period; no struggling.

‡ Gas mixture used, 12½ per cent CO_2, 12½ per cent O_2, 75 per cent air. No struggling during last six periods.

TABLE II.

Effect of Breathing CO_2 on Excretion of Water.

Subject B.

The subject slept on a bed in the laboratory. On waking about 8.15 a.m. he drank 300 to 400 cc. of water; urinated and urine was discarded. No breakfast was eaten. Experiment was started at 8.50 a.m. 100 cc. water were ingested, and urine collected every half hour. Subject lay in bed quietly throughout experiment except for part of time in period 10.20–10.50 a.m.

The time given represents the time when the period was completed.

Time	Urine volume.	Remarks.
1927	*cc.*	
Dec. 3		
9.20 a.m.	130	Subject lying in bed.
9.50 "	300	
10.20 "	200	
10.50 "	120	Gas box over head with air entering.
11.20 "	125	" " " " " " "
11.50 "	220	" " " " " " "
12.20 p.m.	65	CO_2* administered.
12.50 "	75	Air entering box. CO_2 discontinued.
1.20 "	135	" " "

* 11.50–12.12 gas mixture 7 per cent CO_2, 30 per cent O_2, 63 per cent N_2, run into box over subject's head. From 12.12–12.20 the gas mixture was 10 per cent CO_2, 30 per cent O_2, 60 per cent N_2. Symptoms, deep breathing, slight frontal headache, eyes burned a little, legs tired. Symptoms practically gone at 12.30.

Subject G.

Same routine as in previous experiment. Subject lying in bed.

Time.	Urine volume.	Remarks.
1927	*cc.*	
Dec. 3		
11.45 a.m.	145	
12.15 p.m.	135	Air administered through box over head.
12.45 "	158	" " " " " "
1.15 "	90	CO_2† " " " " "
1.45 "	130	Air " " " Respirations, 15–20 per min.
2.15 "	110	Air administered through box.

† 10 per cent CO_2, 30 per cent O_2, 60 per cent N_2 administered. Symptoms as in previous experiment. 12.55, respirations 30 per minute; 1.05, respirations 30 per minute, pulse 60 per minute.

TABLE II—*Concluded.*

Subject N.

Same routine as in previous experiments. Subject lying in bed.

Time	Urine volume.	Remarks.
1927	*cc.*	
Dec. 10		
9 45 a.m.	65	
10 15 "	215	
10.45 "	150	
11.17 "	60	CO_2* administered through box.
11.47 "	15	Box removed at 11.17; breathed air.
12 17 p.m.	235	
12.47 "	125	
1.17 "	175	

* 10 per cent CO_2, 30 per cent O_2, 60 per cent N_2, administered from 10.53–11.17. 12 05, respirations deep, 24 per minute. 12.15 respirations deep, 10 per minute. Symptoms as in previous experiments.

formed. The difficulty in carrying out uncomplicated experiments is evident from a result such as that shown in Period 3 of the first experiment, when a low urine volume resulted from breathing air through the mask. But the absence of such effect in all the other periods of the experiments and the great diminution of urine volume when 12.5 per cent or more of CO_2 was breathed demonstrate the effectiveness of the high concentrations of CO_2. In Period 7 of the first experiment 10 per cent CO_2 caused no outstanding change in urine volume. Similar results were obtained on two other occasions with 10 per cent CO_2 and once with 3 per cent CO_2 in experiments not reported in detail.

Experiments of a similar sort were carried out on human beings. Three dental students were subjects. The procedure used was the same as that employed in the experiments reported previously (Simpson and Wells (1)). A small tight wooden box open at one end with a place cut out for the neck was placed over the subject's head as he lay in bed. The gas mixtures were run from tanks into the box.

The results of the experiments are recorded in Table II. In each case, breathing from 7 to 10 per cent CO_2 caused a marked decrease in the volume of urine excreted. In two experiments

the effect lasted into the 30 minute period, following the CO_2 administration. Normal urine volumes were encountered thereafter.

TABLE III.

Changes in Composition of Blood Due to Breathing High Concentrations of CO_2.

	Aug. 1, 1927.		Aug. 5, 1927.	
	Normal.	After CO_2.*	Normal.	After CO_2.*
Whole blood.				
Sp. gr.	1.048	1 047	1.058	1.055
Solids, *gm. per 1000 gm.*	171.5	171 5	207.7	199.3
Water, *gm. per 1000 gm.*	828.5	828.5	792.3	800.7
Chloride, *mM per 1000 cc.*	91.2	97.1	85.5	91.3
Corpuscles, *cc. per 100 cc.*	35.6	35.8	48.5	47.0
Corpuscles.				
Water, *gm. per 1000 gm. corpuscles*	668	672	661	667
Chloride, *mM per 1000 gm. corpuscle water*	81.3	102.5	83.0	96.1
Serum.				
Sp. gr.	1.022	1.022	1.021	1.021
Solids, *gm. per 1000 gm.*	76.4	78.3	75.1	73.5
Water, *gm. per 1000 gm.*	923.6	921.7	924.9	926.5
Chloride, *mM per 1000 gm. serum water*	115.2	115.9	115.8	116.1

Notes for first experiment: "Technique o.k., dog breathed CO_2 mixture until markedly dyspneic." Second experiment: "Deep breathing began at 9.16. Respirations 9.18, 33; 9.27, 51; 9.35, 55; 9.43, 58."

* The gas mixture used was 50 per cent CO_2, 20 per cent O_2, 30 per cent N_2.

In Table III are recorded the data obtained from analyses on dog blood drawn before and after breathing high concentrations of CO_2. These analyses were referred to in the preceding paper (2). It will be noted that after CO_2 inhalation there is an increase in the water of the corpuscles and an increase in the concentration of

chlorides in this water. This is in accordance with the observations of Doisy and Eaton (3) and others on blood equilibrated with varying CO_2 tensions outside the body. The water and chlorides of the serum showed no demonstrable variations. With no evident change in composition of the serum after breathing high tensions of CO_2 there is no simple explanation for the changes found to occur in the volume and composition of the urine.

SUMMARY.

Experiments on animals and human subjects showed that the urine volume was diminished when high concentrations of CO_2 were breathed.

These experiments confirm and extend data previously reported by the author.

The amount of water in corpuscles and the concentration of chlorides in this water were increased during the breathing of high concentrations of CO_2. No changes in the composition of the serum were apparent.

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THE COPPER METABOLISM OF THE RAT.*

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INTRODUCTION.

Through the identification of copper as an active agent in hemoglobin building in the rat by Hart, Steenbock, and associates (1–7), later confirmed by McHargue, Healy, and Hill (8), the rôle of this element in life processes has acquired new and increased importance. Because of this newly discovered function and also because of its general occurrence in food materials (9, 10)—a fact which suggests its functioning in other capacities—it seemed desirable to study the metabolism of this element more extensively than had hitherto been done.

Results of experiments in which copper in large doses was fed to different species of animals over long periods have been reported by several investigators. Their observations differ greatly as to the toxicity of this element. Huber (11) found that copper salts of amino acids given to guinea pigs by various methods—subcutaneously, intramuscularly, and by mouth—produced only negative results. Drummond (12), in studying the deleterious effects of vegetables colored with copper, and Flinn and von Glahn (13), investigating the poisonous effects of metallic copper and of copper salts on white rats and guinea pigs, obtained no toxic effects; that is, they found neither pigment deposition nor cirrhosis in the liver. Mallory and coworkers (14–16) found pigment deposited in the liver of the rabbit, rat, guinea pig, and monkey when large amounts of copper were fed, and believe that there is a direct relation between pigment cirrhosis and copper poisoning.

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These investigators consider that the liver of a rabbit suffering from chronic copper poisoning undergoes changes comparable in many ways with modifications in the human liver due to a chronic disease known as hemachromatosis.

The data presented in this paper deal with the metabolism of copper in the animal organism; more specifically they deal with the assimilation, storage, transmission to the offspring, and excretion of copper by the rat. For convenience the different experiments are grouped under the various headings that follow.

Effect of Additions of Copper to the Ration on the Copper Content of the Rat.—The first series of experiments deals with the determination of the copper stored by animals produced on two different rations, at various stages in their development. One group of animals came from parents which received no other copper than that contained in the modified stock ration described by Waddell and Steenbock (17). The animals of the second group were the offspring of animals which had been fed a stock ration, essentially the same as the first, plus copper. This stock ration differed from the first in that dried milk instead of fresh milk was used. It was composed of:

	per cent
Yellow corn	50.0
Klim milk powder	33.4
Linseed oil meal	10.5
Crude casein	3 5
Alfalfa	1.4
NaCl	0.4
Bone ash	0.7

and contained 3.29 mg. of copper per kilo. With certain groups a higher copper intake was obtained by feeding 5 mg. of copper daily to each animal. This was done by evaporating a solution of copper sulfate of known copper content on a portion of the stock ration. The material was dried thoroughly, pulverized, and incorporated into the remainder of the ration at the time of feeding. This procedure was followed to avoid any possible deleterious effects of the copper on the major part of the ration. It also insured uniform distribution of the copper throughout the feed and gave sufficiently accurate control of the copper intake.

The female rats from which the young for these experiments

were obtained were placed in cages provided with shavings about 2 days prior to parturition. About one week after the birth of the young the size of each litter was reduced to six rats. A deviation from this general procedure was made in obtaining from copper-fed mothers the young used for analysis at birth. The mothers were kept on ½ inch mesh screens through which the young were allowed to drop and were thus prevented from suckling or coming in further contact with their mothers.

All animals from the copper-fed colony were washed three times with warm water redistilled from glass and the washings were discarded. With the few exceptions indicated in Tables I and V the intestinal tracts of all animals over 13 days of age were removed and discarded. The samples were then dried in a steam oven at 100° and subsequently analyzed for copper.

The copper analyses were made by the methods outlined by Elvehjem and Lindow (18). In all samples representing animals more than 12 days old, the copper was separated from the ash by means of hydrogen sulfide, prior to the development of the color compound. This was necessary because of the large amount of calcium phosphate present.

The special technique described by Waddell, Steenbock, Elvehjem, and Hart (5) was followed in taking the blood samples and the hemoglobin determinations were made with the Newcomer hemoglobinometer.

In Table I are shown the analyses of rats at different ages produced from females fed the stock rations. The figures show that there was a continuous storage of copper as the animal increased in age. The daily storage was about 0.001 mg. of copper for the first 12 days and after that about 0.002 mg. per day. This increased storage after 12 days was probably due to the higher intake of copper, since at this age the young not only received the mother's milk but also began to eat some of the stock ration. In a group of animals of the same age the largest ones usually, though not always, contained the most copper. While the absolute amount of copper per animal increased with age, the percentage decreased continuously up to 85 days, and then increased slightly at 210 to 240 days. This inverse relation between the absolute quantity and the percentage figure in the early life of the rat is similar to that reported for iron by Smythe and Miller (19).

However, the percentage of copper did not increase rapidly after the suckling period as was the case with iron.

In Table II are presented the data obtained from rats produced by the copper-fed females. By comparing the figures in Table I with those in Table II, it may be seen that the copper content of the young cannot be increased by subjecting the mother to a high copper intake.

TABLE I.

Copper Content of Rats at Different Ages (Stock Ration).

Age of animals.	No. of animals.	Moisture.		Dry matter per animal.		Copper per animal.		Copper in dry matter (average).	Hemoglobin.	
		Limits.	Average.	Limits.	Average.	Limits.	Average.		Limits.	Average.
days		*per cent*	*per cent*	*gm.*	*gm*	*mg.*	*mg.*	*mg. per kg.*	*gm. per 100 cc.*	*gm. per 100 cc.*
0	99	81.5–86 7	84.1	0.84–1.20	0.98	0.0089–0.0134	0.0108	11.19		
7	30		80.0		2 38		0.0181	7.97		
12	18	74.0–74.4	74 2	6.06–6.90	6 50	0 0251–0.0269	0.0258	3.97		
26–27 Intestines included.	11	70.1–72.5	71.4	15.40–20.60	17.20	0.0579–0.0688	0.0648	3 82		
22–26	12	61.0–69.5	65.4	14.00–19.50	16.50	0.0448–0.0642	0.0539	3.27	8.53–11.05	9.68
83–85	11	61.9–66.9	63.3	52.00–116.00	79.80	0.1481–0.2500	0.1839	2.39	11.80–14.80	13.25
210–240	10				140.48		0.4422	3.15		

An attempt to produce an opposite effect, *i.e.* to reduce the copper content of the young, was also made. It appeared possible that, if the mother's body was low in copper at the time of the birth of the young (as a small amount of our unpublished data seemed to indicate) subsequent litters produced from such a female on a milk diet might contain a lower quantity of copper. Five stock females which had just given birth to litters and one male were placed on whole milk. Unfortunately, these females

failed to reproduce during a period of 4 months; one died and all showed signs of poor health. It is possible that on a whole milk diet, which is very low in copper, the females were unable to restore the copper reserves of their bodies and that this acted as a limiting factor in reproduction.

TABLE II.

Copper Content of Rats at Different Ages Produced by Females Receiving a High Intake of Copper (Stock Ration Plus 5 Mg. of Copper per Animal Daily).

Age of animals.	No. of animals	Moisture.		Dry matter per animal.		Copper per animal.		Copper in dry matter (average).	Hemoglobin.	
		Limits.	Average.	Limits.	Average.	Limits.	Average.		Limits.	Average.
days		*per cent*	*per cent*	*gm.*	*gm.*	*mg.*	*mg.*	*mg. per kg.*	*gm. per 100 cc.*	*gm. per 100 cc.*
0	67	84.9-87.5	86.4	0 64-0.78	0.72	0.0071-0.0086	0.0078	10.68		
12-13	22	76.8-77.9	77.2	3.86-4.87	4.53	0.0281-0.0310	0.0298	6.62		
25	18	68.9-75.0	71 2	11.00-13.60	12.20	0.0522-0.0652	0.0600	4 94	7.74-9.52	8.82
31	3	71.4-72 2	71.9	14.00-15 00	14.50	0.0871-0.1040	0.0944	6 50	10.94-13.99	11.99
76-78*	8	63 2-68 0	65 7	55.50-73 00	64.86	0.3521-0.6452	0.4595	7.18	9.44-14.49	12.79
210-240†	9				130 14		1 4258	10 96		

* Stock rats fed the stock ration plus 1 mg. of copper per animal daily after 3 weeks of age.

† Adult males fed the stock ration plus 5 mg. of copper per animal daily for last 46 days.

At 12 days, the young rats from the copper-fed mothers, although weighing less, contained about 0.004 mg. more copper per animal than those from the stock females. That this increase indicates a higher copper content in the milk of the copper-fed mothers than that of the stock colony females is questionable. Other data from this laboratory show that the copper content of cow's and goat's milk cannot be influenced by oral administration of copper sulfate. In view of these findings, it is more logical to

attribute the increase of copper in the 12 day young to external contaminating sources, *e.g.* the mother's teats.

At about 13 days of age, the young rat opens its eyes and commences to partake of solid food. The young with the copper-fed mother certainly consumed some of the dam's ration and were thus enabled to store copper. The amount assimilated, however, from the 13th to the 25th day was very small, and the increase in copper content of these animals over that of the stock animals of the same age was less than 0.01 mg. per rat.

The storage of copper is strikingly shown by comparing the two groups at 75 to 85 days of age. The copper-fed animals contained twice as much copper as the animals fed on the stock ration alone.

It may be seen from Tables I and II that the hemoglobin titer of the rats was not increased by adding copper to the stock ration.

Distribution of Copper in Tissues.—As the preceding results showed that the copper content of the whole animal was markedly increased by adding copper to an already adequate ration, the various tissues of the body were analyzed to determine the storage centers.

One group of nine adult rats was fed the stock ration plus 5 mg. of copper per animal daily, while another group of ten animals received only the stock ration. At the conclusion of the feeding period (46 days), the copper-fed animals were washed with water three consecutive times to remove adherent copper salts and the intestinal tracts of all the animals were removed and discarded. After dissection the tissues were dried in a steam oven at 100°. Only part of the bone and muscle tissues were analyzed, but on the basis of a ratio of 33.5 of bone to 39 of muscle, as determined by careful dissection of two stock animals of the same age, the distribution of copper in the bone and muscle tissues of the two groups was calculated. The data for these two groups of animals are given in Tables III and IV.

In the first group, the skin contained the largest quantity of copper. Next in order came the bone, muscle, and certain visceral organs, with approximately equal amounts. Comparison of the data in Table IV with those in Table III shows that copper feeding increased the copper content of bone, kidney, spleen, and liver. The liver showed the most striking increase; in the copper-fed

animals it contained 20 times as much copper as it did in those fed only the stock ration. The spleens, kidneys, and bones of the copper-fed animals contained 5, 2, and 1.6 times as much copper as the same tissues taken from animals raised on the stock ration alone.

All of the above tissues were, of course, not freed from blood. They were analyzed as removed from the dead animal with whatever blood was contained in them. To determine the copper content of blood, other animals that had been fed the same rations

TABLE III.

Distribution of Copper in Tissues of Adult Rats (Stock Ration) (Age of Animals, about 7 to 8 Months).

Tissue.	Moisture.	Sample (10 animals).		Copper per animal.	Copper in dry matter.
		Weight, dry.	Copper.		
	per cent	*gm.*	*mg.*	*mg.*	*mg. per kg.*
Bone	30.6	401.50	1.004	0.1004	2.50
Brain	76.2	4 05	0.037	0.0037	9.14
Heart	80.6	2.52	0.025	0.0025	9.92
Kidney	74.4	9 21	0.114	0.0140	12.41
Liver	72.6	51 05	0.584	0.0584	11.43
Lung	77.0	8 05	0.050	0.0050	6.21
Muscle	70.3	468.00	0.936	0.0936	2.00
Skin	51.7	452.00	1.614	0.1614	3.57
Spleen	75 6	2.93	0.010	0.0010	3.41
Testicle	85.9	5 50	0.048	0.0048	8.65
Totals		1404.81	4.422	0.4422	3.15

were used. The blood of animals fed the stock ration was found to contain 0.0543 mg. of copper per 100 cc. The blood of rats fed the stock ration plus 5 mg. of copper per animal daily for 9 days, contained 0.0821 mg. of copper per 100 cc. The livers of this last group were also analyzed to obtain confirmatory data and were found to contain 0.4234 mg. of copper per animal. In 9 days these animals had stored about one-half as much copper in their livers as the quantity present in the same organ taken from rats fed on copper for 46 days. This comparison indicates that the retention of copper takes place rapidly but not exclusively during the first few days of copper feeding.

Experiments with Milk as Basal Diet.—In these experiments young rats, produced both from stock females and from copper-fed females, were placed, when 3 to 4 weeks old, in groups of five or six in cages provided with screens. The basal diet was whole milk produced by cows fed the wheat straw ration described in a previous paper (5). The copper content of this milk was 0.16 mg. per kilo. Where copper was used as a supplement to the

TABLE IV.

Copper Content of Tissues of Adult Rats Receiving High Intake of Copper (Stock Ration Plus 5 Mg. of Copper per Animal Daily for Last 46 Days).

Animals were about 7 to 8 months of age.

Tissue.	Moisture.	Sample (9 animals). Weight, dry.	Sample (9 animals). Copper.	Copper per animal	Copper in dry matter.
	per cent	*gm.*	*mg.*	*mg.*	*mg. per kg.*
Bone	31.2	349.00	1.483	0.1648	4 25
Brain	76 1	3.82	0.043	0.0047	11 16
Heart	75 9	2 53	0.031	0 0034	12 25
Kidney	72.9	8.40	0.229	0.0254	27.20
Liver	74 2	40 50	8.640	0.9600	213 32
Lung	78.6	6 00	0.064	0.0071	10.67
Muscle	69 0	401 00	0.842	0.0936	2 10
Skin	58 5	353 00	1.412	0.1569	4.00
Spleen	70 4	2 52	0.044	0 0049	17.47
Testicle	85.7	4 50	0.045	0.0050	10.00
Totals		1171 27	12 833	1.4258	10 96

milk, a copper sulfate solution equivalent to 1 mg. of copper per animal per day was added directly to the milk.

The data in Table V show the effect of a milk diet and of milk plus copper on the copper content of the animals.

In Table I it was shown that rats about 3 weeks old contained 0.0539 mg. of copper. On a milk ration, at 61 to 76 days of age the animals had the same copper content as at 3 weeks and showed the characteristic low hemoglobin titer reported by Waddell and associates (5).

The animals fed milk supplemented by 1 mg. of copper per

animal daily contained, at 77 to 83 days, almost 4 times as much copper as those of the preceding group, and about the same quantity as rats of similar age fed the stock ration. The average figure, 0.189 mg., was not one-half as high as the level in the rats fed the stock ration with copper additions. However, it should be noted that the rats on milk and copper did not weigh half as much as those analyzed at a corresponding age which had been fed on the stock ration plus copper. Consequently, the figures show that per unit of weight, the two groups contain about the same quantity

TABLE V.

Copper Content of Rats on Milk with and without a Copper Supplement.

Age of animals		Moisture		Dry matter per animal		Copper per animal		Copper in dry matter (average)	Hemoglobin	
		Limits	Average	Limits	Average	Limits	Average		Limits	Average
Whole milk										
days		*per cent*	*per cent*	*gm*	*gm*	*mg*	*mg*	*mg per kg*	*gm per 100 cc*	*gm per 100 cc*
61-68 Intestines included	6	70.8-72.4	71.7	21.00-30.00	24.75	0.0455-0.0555	0.0521	2.11	4.24-5.18	4.71
76	5	65.7-68.3	67.1	26.00-34.00	28.70	0.0500-0.0666	0.0559	1.95	3.72-6.45	5.25
Whole milk plus 1 mg. copper per animal daily.										
77-83	12	61.5-66.7	64.9	25.50-37.00	30.10	0.1575-0.2469	0.1890	6.36	8.67-15.46	10.98

of copper. The rats on milk and copper had an average hemoglobin titer of 10.98, a figure which, due to the low iron content of the milk, is below normal.

The experiment of which the results are pictured graphically in Chart I was carried out in order to determine the effect of subjecting the dam to a high copper intake on the resistance of the young to anemia. Earlier work by Waddell and Steenbock (unpublished) in which 10 per cent of dried beef liver had been incorporated in the diet of mature females, had shown that the young

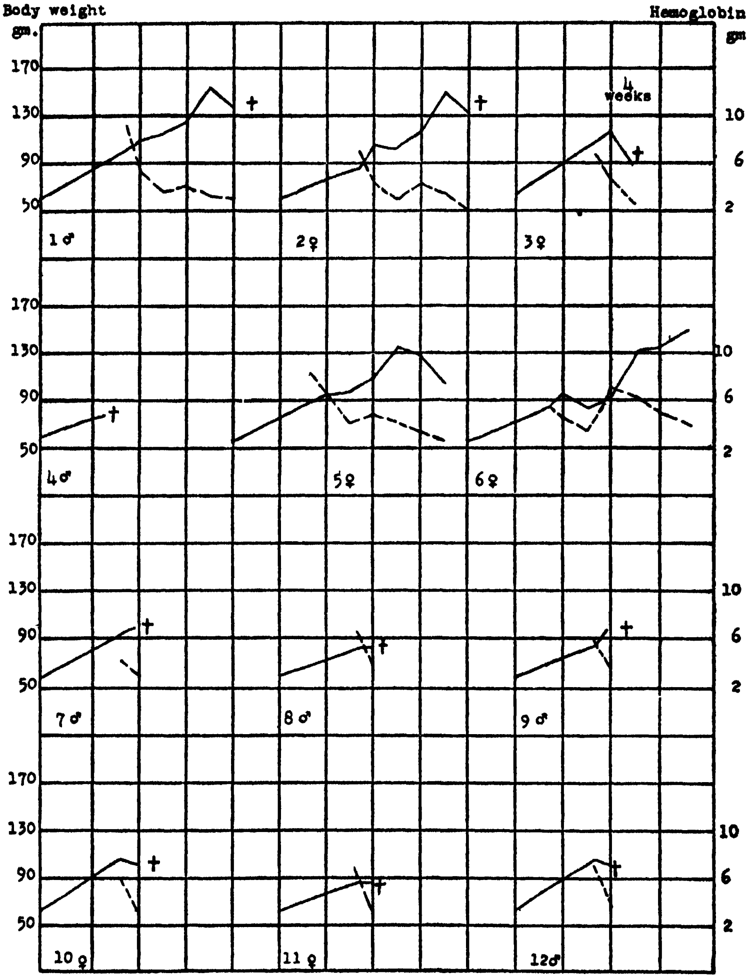

Chart I. Feeding 5 mg. of copper daily to the mother rat during the gestation period and until the young were 12 days old did not prevent anemia in the young when put upon a whole milk diet at 28 days of age. The life span and the hemoglobin values in these animals were no greater than those of young rats produced from females fed the stock ration as reported in the literature (5). In this and the following charts, the solid line represents body weight, the broken line, hemoglobin values in gm. per 100 cc. of blood. The dagger at the end of the curve denotes death.

produced by these females were no better able to maintain their hemoglobin levels on a milk diet than young from the stock colony. It was thought best, however, to repeat this work with only copper as a supplement. Each female received 5 mg. of copper daily for 3 months prior to, and 12 days following, the birth of the young. The females with their litters, each reduced to six, were then transferred to clean cages and had access to the stock ration only, until the young, when 28 days old, were placed on milk. The hemoglobin curves show that all of these rats became anemic and that the life span of the majority of them was no greater than that of stock rats used in similar experiments (5). These results serve as additional evidence for the conclusion drawn from the analytical data; *viz.*, that the copper content of the young rat is not influenced to an appreciable extent, if at all, by maintaining the dam on a high copper intake during the gestation and suckling periods. Four of these rats, Nos. 1, 2, 5, and 6, lived 7 to 13 weeks longer than their mates. We believe that this was due to copper contamination from Rats 24 to 29 which had been moved to the cage adjoining that occupied by Rats 1, 2, etc. at the end of the 10th week. 2 weeks later the hemoglobin values of Rats 1, 2, 5, and 6 had risen considerably. At first this puzzling effect could not be explained. When it was suspected that the copper-fed animals were transmitting copper to their neighbors, a sheet of metal was inserted between the two groups. The hemoglobin values of the four milk-fed animals then began to fall; two died after 5, and the other two after 11 weeks.

Chart II gives the hemoglobin and body weight curves of eleven rats produced from females fed copper for 3 months prior to, and for 29 days following, the birth of the young. At this age, the young were placed on a milk diet. These animals, though showing the gradual progress of anemia, lived longer than most of those of the immediately preceding experiment, probably because of the copper obtained from the mother's ration and stored in the tissues and intestinal tract between the ages of 12 and 29 days. This copper was utilized in maintaining a low plane of hemoglobin synthesis over an extended period of time.

In Chart III are shown the positive controls for the preceding groups. These stock colony animals received milk plus 1 mg. of

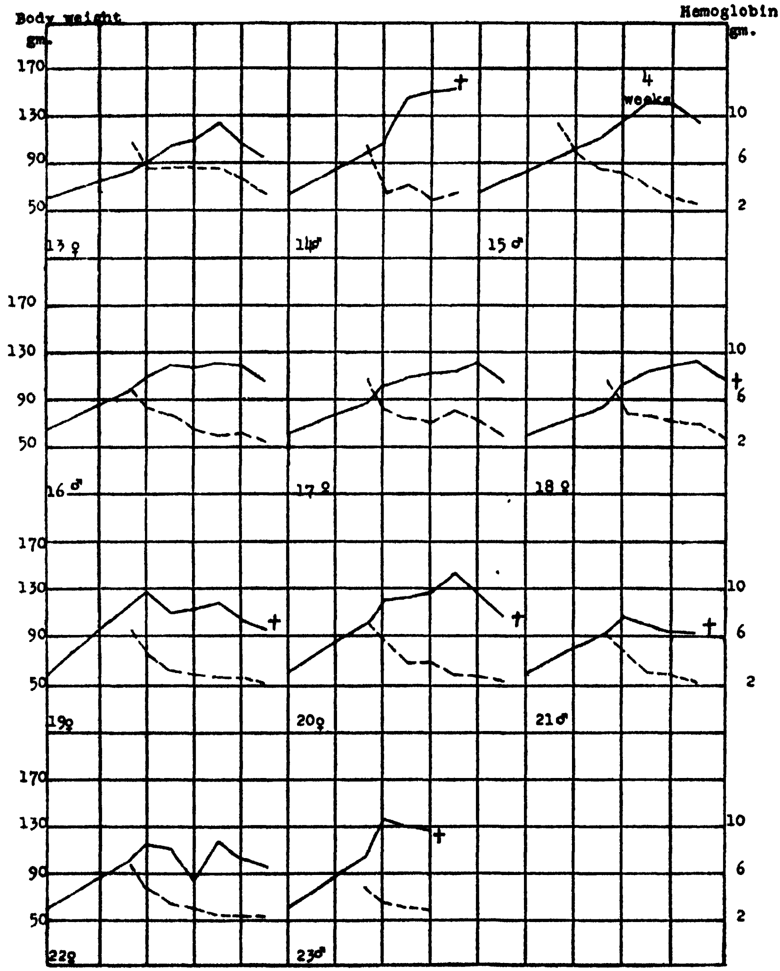

Chart II. In contrast with the results in Chart I, feeding the same amount of copper to the mother rat until the young were 29 days old increased their span of life and delayed the fall in hemoglobin. Between the 12th and 29th days the young consumed some of the mothers ration, stored copper, and thus increased their resistance to anemia.

copper per animal daily. Over a period of 18 weeks, there was a constant increase in growth, and the hemoglobin values, although below normal, remained quite uniform. Unpublished data from this laboratory show that young rats maintained on milk sup-

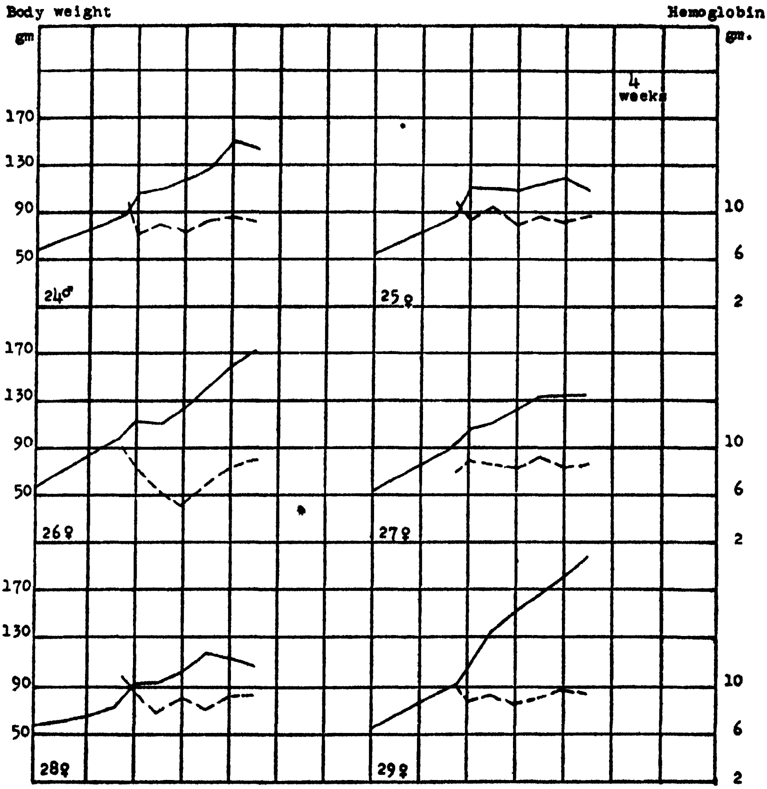

Chart III. Supplementing the milk diet of young rats with 1 mg of copper daily maintained their hemoglobin values and prevented death even when the young were produced by mothers fed on the stock ration without copper additions.

plemented with small amounts of copper exhibit for a long time a marked anemia due to the low iron content of the diet. They eventually overcome this anemia.

Excretion of Copper.—Since the preceding experiments had

shown that copper is rapidly stored, it seemed desirable to study the excretion of this element. A metabolism cage large enough for one rat was constructed out of an 8 × 12 inch glass jar. In the jar were placed three sheets of filter paper to absorb the urine, a $\frac{1}{4}$ inch mesh screen to catch the feces, and a $\frac{1}{2}$ inch mesh screen for

TABLE VI.

Record of Copper Balance of Rat 1.

Date.	Dried feces.	Feces, copper.	Urine, copper.	Percentage of total copper in feces.	Intake, copper.	Output, copper.	Balance.
			Pre-copper period.				
1929	*gm.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
Feb. 8–13	12.86	0.1667	0.0899	64.9	0.2350	0.2566	−0.0216
" 14–19	14.88	0.1430	0.0821	63.5	0.2350	0.2251	+0.0099
			Transition period.				
Feb. 20–25	16.00	24.1800	0.4444	98.2	30.2350	24.6244	+5.6106
			Copper period.				
Feb. 26–Mar. 3	16.67	26.7641	0.4348	98.4	30.2350	27.1989	+3.0361
Mar. 4–9	14.89	29.8507	0.4286	98.6	30.2350	30.2793	−0.0443
			Transition period.				
Mar. 10–15	16.28	4.3216	0.3158	93.2	0.2350	4.6374	−4.4024
			Post-copper period.				
Mar. 16–21	14.46	0.3333	0.2890	53.6	0.2350	0.6223	−0.3873
" 22–27	15.45	0.2261	0.1798	55.7	0.2350	0.4059	−0.1709
" 28–Apr. 2	15.68	0.1320	0.1053	55.6	0.2350	0.2373	−0.0023
Apr. 3–8	15.40	0.1565	0.0770	67.0	0.2350	0.2335	+0.0015

the support of the animal. A feed cup, water tube, and cover completed the equipment.

Three adult male rats, one in each cage, were used in this experiment. 12 gm. of the stock ration were fed each animal daily, and water redistilled from glass was given *ad libitum.* The feeding periods were really three in number, although classified as five in Tables VI and VII. There was a 12 day period on the stock ration, an 18 day copper feeding period, and a 30 day post-

copper period. Because of the overlapping of ingestion and excretion, the first 6 days of the last two periods have been designated as transition periods. Urine and feces were collected at 3 day intervals. At the conclusion of the copper feeding period the animals were washed three times with redistilled water to remove adhering particles of the copper ration from the fur, and

TABLE VII.

Record of Copper Balance of Rat 3.

Date.	Dried feces.	Feces, copper.	Urine, copper.	Percentage of total copper in feces.	Intake, copper.	Output, copper.	Balance.
			Pre-copper period.				
1929	*gm.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*
Feb. 8–13	13.69	0.1765	0.0832	67.9	0.2350	0.2597	−0.0247
" 14–19	16.08	0.1682	0.1089	60.7	0.2350	0.2771	−0.0421
			Transition period.				
Feb. 20–25	17.24	24.6410	0.4143	98.3	30.2350	25.0553	+5.1797
			Copper period.				
Feb. 26–Mar. 3	15.05	25.9071	0.5000	98.1	30.2350	26.4071	+3.8279
Mar. 4–9	17.37	30.6121	0.4615	98.5	30.2350	31.0736	−0.8386
			Transition period.				
Mar. 10–15	15.12	3.8242	0.2643	93.5	0.2350	4.0885	−3.8535
			Post-copper period.				
Mar. 16–21	17.56	0.2264	0.2222	50.5	0.2350	0.4486	−0.2136
" 22–27	16.35	0.1667	0.1565	51.6	0.2350	0.3232	−0.0882
" 28–Apr. 2	15.35	0.1558	0.0988	61.2	0.2350	0.2546	−0.0196
Apr. 3–8	15.48	0.1620	0.0920	63.8	0.2350	0.2540	−0.0190

the washings were saved for analysis. The jars, screens, etc., were also carefully cleaned and the collected material added to the feces of the last copper period.

In Tables VI and VII are given the analytical data for two of the rats. The samples from the third animal were not analyzed because the rat became ill subsequent to washing and refused to eat normally for several days. The data show that in normal

metabolism approximately 2 parts of copper are excreted in the feces to 1 part in the urine. In the period when copper was fed, about 98.5 per cent of the copper was eliminated in the feces while the quantity in the urine represented a marked increase over that present in the pre-copper period. In the post-copper period the stored copper was gradually eliminated and the animal reached a normal level of copper excretion only after 4 or 5 weeks. Elimination of copper in the urine seems to have been more gradual than in the feces and this fact leads one to believe that the copper actually stored may have been largely excreted in the urine.

As given in Table IV, the relatively small amount of copper in the kidney compared with that in the liver shows that there is no kidney injury preventing its excretion, nor is there permanent combination of copper with the liver tissue that might prevent its excretion. What is true of the liver is true of the other organs, although to a lesser degree. The liver is mentioned in this particular connection because it is the organ in which copper accumulates to the greatest extent. This conclusion was further confirmed by the analysis of the livers of these rats 53 days after the last copper was fed. The livers of Rats 1 and 3 contained 0.0597 and 0.0666 mg. of copper respectively, values which approach closely the average given in Table III.

The total copper intake of each rat was 92.4 mg. The recovery of copper from Rat 1 was 89.56 mg., while that from Rat 3 was 89.28 mg. These figures include the copper of the wash water which averaged 0.84 mg. per animal. These data show that about 97 per cent of the copper in the ration can be recovered in the excreta and also indicate a high degree of accuracy for the analytical method (18).

We have found only two papers in the literature that pertain to the excretion of copper. Filehne (20) reported that about 98 per cent of the copper administered as "cupratin" to cats and dogs was eliminated in the feces. Flinn and von Glahn (13) state that with twelve young rats that received the equivalent of 1 mg. of copper as copper chloride daily, the individual average daily copper excretion over a period of 7 weeks was 0.97 mg. in the feces and 0.016 in the urine.

SUMMARY.

1. The absolute amount of copper in the body of a rat on an adequate ration gradually increased from 0.0108 mg. at birth to 0.4422 mg. at 210 to 240 days. On a percentage basis there was a constant decrease from birth up to 85 days and a slight increase at 210 to 240 days.

2. The copper content of a rat at birth was not raised by subjecting the mother to a high copper intake. There was no indication of increased placental transmission, nor was there increased transmission of copper through the milk.

3. Feeding of additional copper led to marked increases in the absolute copper content and the percentage of this element present in the bodies of rats of various ages fed different rations. This increase was most striking in the more adult animals which at 75 to 85 days of age contained 2 and at 210 to 240 days 3 times as much copper as the animals fed on the stock ration alone. The percentage figure increased continuously after 25 days.

4. The distribution of copper in the tissues of two groups of adult rats, one fed the stock ration, the other the stock ration supplemented with copper, was determined. Copper feeding increased the copper content of the skeleton, the kidney, the spleen, and the liver, 1.6, 2, 5, and 20 times, respectively. The copper content of the blood from adult rats fed the stock ration was 0.0543, while that from the copper-fed animals was 0.0821 mg. per 100 cc.

5. The copper content of rats on an anemia-producing diet was the same at 3 weeks and at the time of death (9 to 11 weeks).

6. While the young from copper-fed females have no increased resistance to anemia at birth, they can acquire some resistance during the period between 12 and 29 days through ingestion of the mother's ration.

7. Adult rats fed the stock ration excreted 2 parts of copper in the feces to 1 part in the urine. When the stock ration was supplemented with copper, about 98.0 per cent of this element was excreted in the feces. The urine copper increased to about 5 times the quantity that was present in the pre-copper period. There was a storage of copper during the copper feeding period. This stored copper was eliminated during 4 or 5 weeks of post-period feeding.

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THE NATURE OF THE ACIDS PRODUCED IN THE FERMENTATION OF MAIZE BY CLOSTRIDIUM ACETOBUTYLICUM.

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The production of acid is one of the outstanding features of carbohydrate fermentation by the acetone-butyl alcohol microorganism, *Clostridium acetobutylicum*. The major part of the acid is volatile and consists chiefly of acetic and butyric acids. The ratio of these two acids and their rate of production have been investigated with somewhat divergent results by Reilly, Hickinbottom, Henley, and Thaysen (1) and Speakman (2). Traces of formic acid have been reported by Donker (3) as a final product in the fermentation of certain sugars but as absent in the fermentation of corn mash. Traces of caprylic and capric acids were found by Marvel and Broderick (4) in the "yellow oil" residue from the butanol distillation.

The non-volatile acid has not been investigated to the same extent as the volatile portion. Schmidt, Peterson, and Fred (5) isolated *l*-leucic acid from this fraction and Speakman (6) reported the presence of *p*-hydroxyphenyllactic acid. In both reports it was concluded that these acids are deaminization products of leucine and tyrosine respectively.

The several acids previously reported have been obtained from separate fermentations at different times. It was the purpose of this investigation to carry out simultaneously as many of the determinations as possible on the same culture, to make these determinations at more frequent time intervals, to separate the non-volatile acid into an α-hydroxy and a residual portion, and to attempt a correlation of the acidity with the production of the various neutral products.

Analytical Methods.

Acidity.—Titratable acid was determined by heating 10 cc. of sample to the boiling point and titrating with 0.1 N NaOH to phenolphthalein. *Volatile acid* was obtained by acidifying 250 cc. of sample with H_2SO_4 and distilling with steam. The steam distillate was analyzed for *formic acid* by the Fincke method (7) and for *other volatile acids* by the Duclaux procedure as given by Gillespie and Walters (8). The *non-volatile acid* in the residue from the steam distillation was extracted with ether in a Kutscher-Steudel extractor, converted into the barium salts, and freed from fat and other water-insoluble matter by filtering. α-Hydroxy acid was determined in this filtrate by the method of Friedemann, and associates (9, 10). Residual acid was computed by the difference between the non-volatile and the α-hydroxy acid.

Residual Carbohydrate.—Starch and pentosans remaining in the fermented mash were hydrolyzed with 1.4 per cent HCl for 2 hours at 100°. Total reducing sugar was then determined by the micro method of Shaffer and Hartmann as modified by Stiles, Peterson, and Fred (11).

Solvents.—Total solvents were determined by distilling 100 cc. from 500 cc. of fermented mash and taking the specific gravity of the distillate with a pycnometer at 21°/21°. Acetone was estimated by Goodwin's modification (12) of Messinger's method, ethyl alcohol by Bogin's (13) water titration method, and butanol was obtained by difference.

EXPERIMENTAL.

In Experiment 1 40 liters of 5.4 per cent corn mash in a 50 liter Pyrex flask were inoculated with 800 cc. of an actively fermenting culture of *Clostridium acetobutylicum* and allowed to ferment at 37°. In Experiment 2 two 12 liter flasks containing 8 liters of 7.4 per cent corn mash were inoculated with 160 cc. of culture and incubated at the same temperature as in Experiment 1. Samples were taken from one of the flasks at 3 hour intervals. The other served as a control, not opened, and shaken but once, before the 66 hour sample for solvent analysis was taken. In Experiment 3 four 750 cc. Erlenmeyer flasks, each containing 500 cc. of 7.0 per cent corn mash, were also inoculated with 2 per cent of inoculum.

12 hours later an excess of sterile $CaCO_3$ was added to three of these, one remained as a control, and all four were then attached to a rotatory shaking machine which was kept in motion about three-fourths of the incubation period.

In Experiment 4 four different cultures were used to ferment 2 liters of 7.5 per cent corn mash contained in 3000 cc. Erlenmeyer flasks. The inoculum for each flask was 40 cc. Samples were taken just before the acidity peak for volatile acid, and at the end of fermentation for total solvents, volatile acid, and residual carbohydrate. The effect of concentration of corn mash was studied in Experiment 5. The size of flask, the time of sampling, and the analyses were the same as in the preceding experiment. In each of the last two experiments an extra flask was included in order to study the effect and destruction of formic acid added to the fermenting mash after the break in acidity.

Fermentation Products.—The various products of the fermentation are given in Table I. For the sake of convenience in the interpretation of the conversion of corn meal to the fermentation products the results are expressed in terms of percentage of the original dry corn. No data on the amounts of carbon dioxide and hydrogen were obtained in the present investigation, but the quantity of these products was obtained from the work of McCoy, Fred, Peterson, and Hastings (14). These authors report that 43.8 per cent of the corn was converted to carbon dioxide and 1.4 per cent to hydrogen. The gases together with the residual protein, ash, and fat of the corn will account for practically all the difference between the sum of the products obtained and the weight of initial dry corn. A lower yield of solvents was obtained in Experiment 1, but this was probably due to the different culture which was used in this experiment. Except for a slightly low yield of ethanol from Flask 1, the quantities of neutral products obtained from both flasks of Experiment 2 were normal and showed only the usual variation of parallel fermentations by this organism.

The presence of $CaCO_3$ in the fermenting mash did not completely suppress the formation of solvents but allowed a 16.4 per cent yield of solvents from the corn, which is equal to 60 per cent of the control yield. This indicates the partial fermentability of the calcium salts. These results are in good agreement with Reilly, Hickinbottom, Henley, and Thaysen (1) who obtained

approximately a 13 per cent yield of solvents from corn in a similar experiment. The control flask of the calcium carbonate series returned an average yield of total solvents and of butanol, but showed a slight shifting of acetone to ethyl alcohol as compared to the control of Experiment 2. By comparison of the products from the flasks to which $CaCO_3$ was added with either of these controls, it is evident that the effect of the neutralizing agent on the solvents was to decrease the percentage of ethyl alcohol and acetone and to increase that of butanol. Data on the distribution of solvents in all the experiments, given in Table VIII, establish this point. On the assumption that acetaldehyde is an intermediate product, this would indicate that the calcium salts in the fermenting mash catalyzed the formation of aldol from acetaldehyde, as other weak alkalies are known to do, and resulted in more butyric acid and consequently butyl alcohol. The hypothesis that acetic acid is converted to acetoacetic acid and thence to acetone with the loss of CO_2 is substantiated by the fact that the sum of the volatile acids and the solvents obtained in Experiment 3 exceeded the weight of solvents of the control of this series. (A correction must be made for the loss of 14 in the molecular weight of the butyric acid convertible to butanol.) Conversion of the intermediate acids to neutral products must involve the liberation of gas.

Production of Acid throughout the Fermentation Period.—Amounts of the various acids remaining at the end of the active period of fermentation in Experiments 1 to 3 are recorded in Table I. Flask 2 of Experiment 2 was sampled at the 84 hour period for convenience in analysis. There is but little difference in the acid content between 66 and 84 hours. Fluctuations in the amounts of titratable, volatile, and non-volatile acid at the progressive time intervals, beginning at 6 hours, are given in Chart 1. The curves for the non-volatile acid of the two experiments are almost identical. Those for the titratable and volatile acid of Experiment 1, however, show a trifle longer acidity period with less volatile acid at the peak, and more volatile acid in the final stages. These differences in the first 24 hours of the fermentation are not unusual. The increased volatile acid at the end of the first experiment affords a partial explanation of the decreased yield of solvents.

Nature of the Volatile Acid.—As the work of Reilly, Hickinbot-

tom, Henley, and Thaysen (1) and Speakman (15) indicates that acetic and butyric are the only volatile acids present in significant proportions, no preliminary fractionation of the steam distillate

TABLE I.

Fermentation Products of Clostridium Acetobutylicum.

	Experiment 1, Culture C.		Experiment 2, Culture A.				Experiment 3, Culture A.			
			Flask 1.*		Flask 2.†		Excess $CaCO_3$.		Control.	
Concentration of dry corn in mash, *per cent*	5.4		7.4		7.4		7.0		7.0	
	hrs.	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*
Total solvents	66	23.15	66	27.70	66	27.11	92	16.40	92	27.30
Butanol		12.73		17.14		16.35		11.00		16.33
Acetone		7.48		9.20		8.92		4.74		8.16
Ethanol		2.94		1.36		1.84		0.66		2.81
Total acid	63	4.92	66	2.38	84	2.30	92	16.34		
Volatile		3.82		1.45		1.41		15.00		
Formic				0.05		0.05		0.07		
Acetic		1.72		0.96		0.96		6.31		
Butyric		2.10		0.44		0.40		8.62		
Non-volatile		1.10		0.93		0.89		1.34		
α-Hydroxy‡				0.37		0.37		0.62		
Residual§				0.56		0.52		0.72		
Residual carbohydrate	63	7.96	66	8.61	66	8.06	92	7.36		
Sum of products and remaining carbohydrate		36.03		38.69		37.47		40.10		

* This flask was sampled at 3 hour intervals.

† Control flask which was not sampled until end of fermentation.

‡ Computed as leucic acid.

§ Computed as β-hydroxybutyric acid.

was made before applying the Duclaux method to the determination of these acids. Formic acid, after being qualitatively identified by the reduction of silver nitrate and mercuric chloride solu-

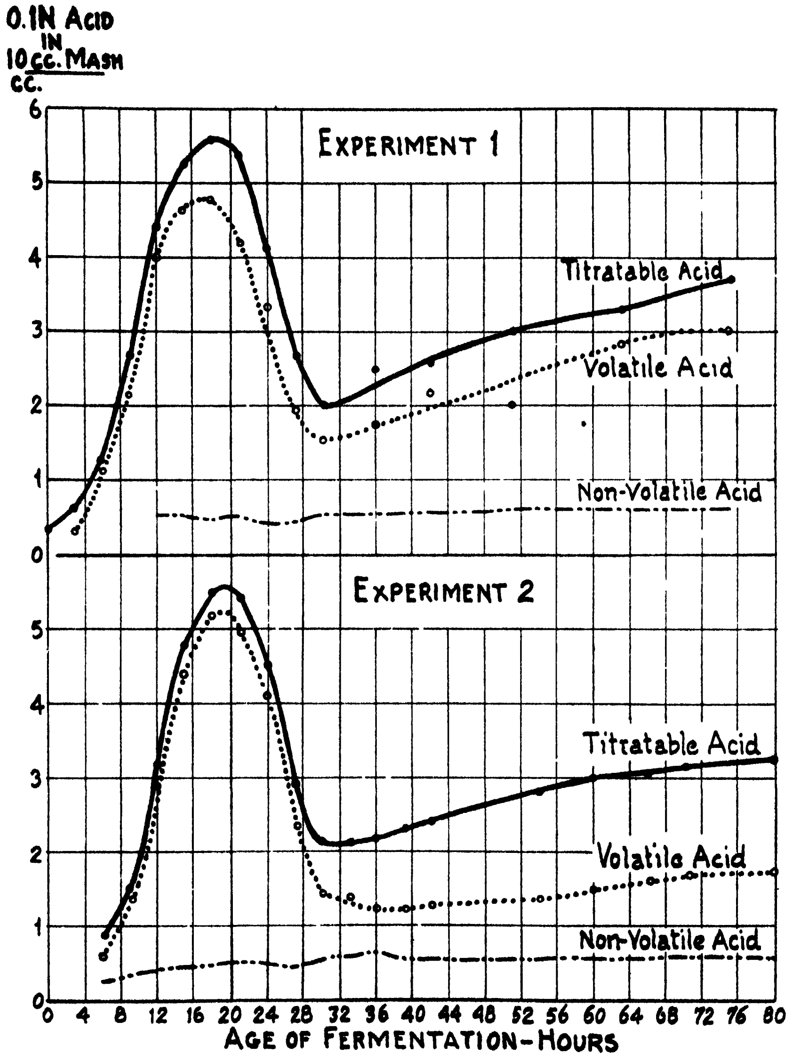

CHART 1. Fluctuations in acid content during the acetone-butanol fermentation.

tions, was determined by Fincke's (7) method, and the amount of this acid present in each fraction was subtracted from the Duclaux data. The amount of acid remaining was then computed to its acetic and butyric values. The distilling constants for the volatile

TABLE II.

Duclaux Distilling Constants of Volatile Acid Produced by Clostridium Acetobutylicum.

Experiments 2 and 3.

Time after inoculation.	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
6	12.3	24.0	35.0	45.4	55.4	64.9	73.9	82.6	91.1	100.0
9	13.1	25.4	37.0	47.8	57.9	67.4	76.2	84.4	92.2	100.0
12	13.4	25.9	37.6	48.5	58.7	68.1	76.9	85.0	92.6	100.0
15	13.0	25.6	37.4	48.4	58.7	68.2	76.9	85.1	92.6	100.0
18	13.2	25.5	37.2	48.0	58.2	67.6	76.4	84.6	92.4	100.0
21	12.9	25 0	36.6	47.4	57.5	67.0	75.8	84.1	92.1	100.0
24	12.5	24.3	35.6	46.2	56.3	65.7	74.7	83.2	91.6	100.0
27	10.8	21.3	31.5	41.4	51.1	60.7	70.1	79.6	89.3	100.0
30	9.7	19.2	28.7	38.2	47.6	57.2	66.9	77.0	87.7	100.0
33	9.6	19.1	28.6	38.0	47.5	57.0	66.8	76.9	87.7	100.0
36	9.9	19.7	29.3	38.9	48.3	57.9	67.5	77.5	88.0	100.0
39	9.4	18.8	28.1	37.5	46.8	56.4	66.1	76.3	87.3	100.0
42	9.4	18 7	28.0	37.3	46.7	56.3	66.0	76.3	87.3	100.0
54	9.8	19.6	29.1	38.7	48.2	57.7	67.4	77.4	88.0	100.0
60	10.1	20.1	30.0	39.6	49.2	58.7	68.3	78.1	88.4	100.0
66	10.3	20.3	30.2	39.9	49.5	59.0	68.5	78.3	88.5	100.0
70	10.4	20.5	30.4	40.1	49.7	59.2	68.7	78.5	88.6	100.0
84	10.4	20.7	30.7	40.5	50.1	59.6	69.1	78.8	88.8	100.0
Control.										
84	10.1	20.0	29.8	39.5	49.0	58.5	68.1	78.0	88.3	100.0
$CaCO_3$										
92	12.9	25.1	36.5	47.3	57.4	66.9	75.7	84.1	92.0	100.0
92	12 9	25.1	36.6	47.3	57.4	66.8	75.7	84.0	92.0	100.0
Formic acid.	6 0	12.5	19.4	27.0	35.2	44.2	54.4	66.3	80.7	100.0
Acetic "	7.8	15.9	24.3	33.0	42.2	51.8	62.0	73.1	85.5	100.0
Butyric "	18.2	34.3	48.4	60.7	71.1	79.9	87.1	92.8	97.1	100.0

acid samples together with data obtained in our apparatus for pure formic, acetic, and butyric acids are presented in Table II. The constants obtained all lie between the values for acetic and butyric acids, but the samples up to the 30th hour of the fermenta-

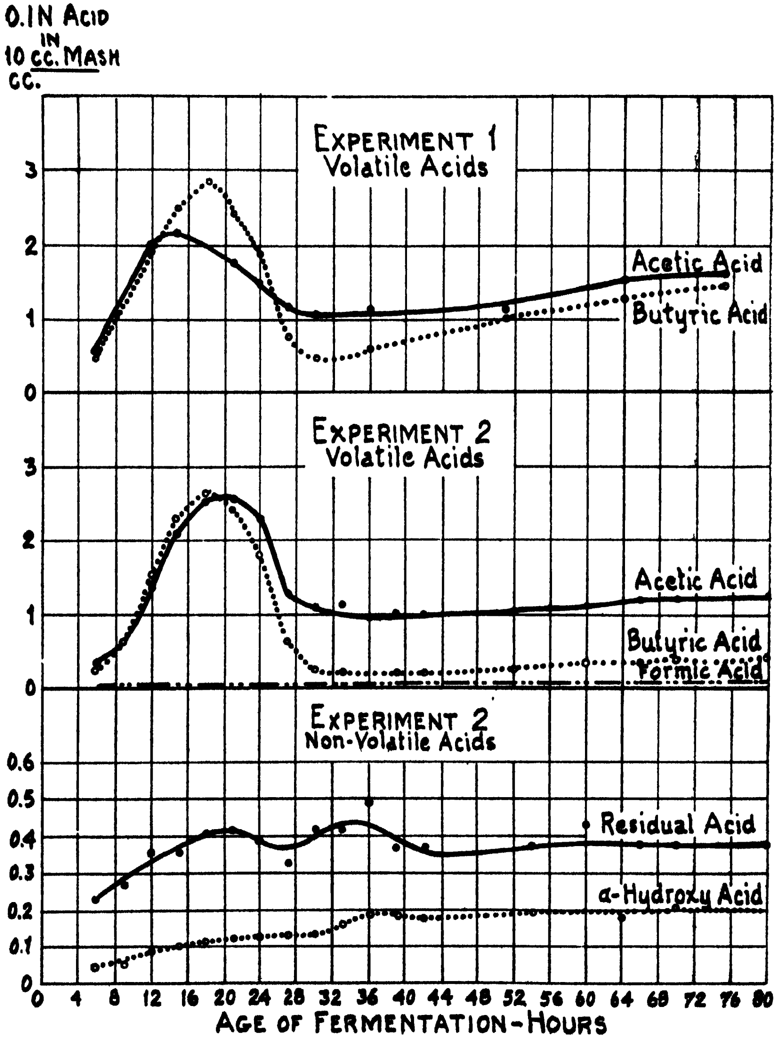

CHART 2. Progressive changes in the quantity of individual acids present throughout the acetone-butanol fermentation.

tion, those after the 54th hour, and also those distilled from the calcium salts indicate the presence of a fourth acid. Reilly *et al.* also report the presence of a volatile acid other than acetic and butyric which may be identical with our fourth acid.

Calculations from the Fincke and Duclaux analyses have made possible a separation of the volatile acid into its three chief constitutents in Chart 2. A difference between the quantities of acetic acid in the two experiments after the 15th hour is at once

TABLE III.

Amounts of Formic Acid Present in Fermenting Corn Mashes of Clostridium Acetobutylicum.

Culture.	Concentration of dry corn in mash.	Age of fermentation.	Weight of HgCl obtained per liter of mash.	Formic acid present in 1 liter of mash.
	per cent	*hrs.*	*gm.*	*cc. 0.1 N*
A	7.40	65	0.476	10.1
B	7.50	17	0.076	1.6
"	7.50	70	0.534	11.3
C	7.50	19	0.077	1.6
"	7.50	70	0.565	12.0
D	7.50	21	0.085	1.8
"	7.50	70	0.645	13.7
A	7.50	23	0.036	1.6
"	7.50	70	0.488	10.3
"	3.26	19	0.057	1.2
"	3 26	64	0.374	8.0
"	5 30	16	0.045	1.2
"	5 30	66	0.401	8.5
"	7.38	21	0.080	1.7
"	7.38	69	0.372	7.9

evident. In Experiment 1 acetic acid apparently was more rapidly converted to solvents than in Experiment 2. After the 30th hour the curves for this acid are very much the same. Over the acidity peaks of the two experiments the production and utilization of butyric acid was similar. From the 30th hour on, however, there was from 2 to 4 times as much butyric acid in the earlier experiment. If butyric acid and butanol come from the same precursor, there should then be less butanol in Experiment 1 than in Experiment 2. This is in accord with the distribution of solvents given later in Table VIII.

Influence of Culture and Concentration of Mash on the Production of Formic Acid.—As the work in Experiment 2 revealed the presence of formic acid, several cultures and three concentrations of corn mash were used to determine the effect of these factors on the production of formic acid. Because of the great amount of work involved with the Duclaux analyses, the times of sampling

TABLE IV.

*Comparison of Solvent and Volatile Acid Products from Various Cultures. Experiment 4.**

Culture	A		B		C		D		A		A†	
Concentration of dry corn in mash, *per cent.*	7.4		7.5		7.5		7.5		7.5		7.4	
	hrs.	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*
Total solvents	65	25.50	70	24.18	70	24.73	70	24.77	70	24.26	70	19.58
Butanol		15.58		14 97		14 76		14.32		14.31		11.36
Acetone		7.91		7.86		7.52		7.80		7.72		6.15
Ethanol		2.10		1.35		2.45		2.65		2.23		2.07
Volatile acid	65	1.91	70	2.07	70	1.83	70	1.68	70	2.09	70	1.90
Formic		0.06		0.07		0.07		0.08		0.06		0.19
Acetic		1.35		1.40		1.38		1.31		1.45		1.38
Butyric		0.50		0.60		0.38		0.29		0.58		0.33
Residual carbohydrate	65		70	9.01	70	9.93	70	9.80	70	8.35	70	22.14

* A different corn-meal was used in this experiment.

† This flask had 12.8 cc. of 0.1 N formic acid added per 250 cc. of mash just after acidity peak.

were limited to one just prior to the acidity peak and another at the end of the fermentation.

The relation of different strains of *Clostridium acetobutylicum* to the accumulation of formic acid in the medium are shown in Tables III and IV.

Culture D contained the largest quantity of formic acid and conversely the smallest quantity of acetic and butyric acids. This inverse relation is probably the outcome of the organism's

attempt to protect itself against an unfavorable hydrogen ion concentration. Since the ionization of formic acid is more than

TABLE V.

Effect of Concentration of Corn Mash on Solvent and Volatile Acid Products. Experiment 5. Culture A.

Concentration of dry corn in mash, *per cent*	3.26		5.30		7.38		7.34*	
	hrs.	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*	*hrs.*	*gm. per 100 gm. dry corn*
Total solvents	64	27.86	66	27.37	69	27.25	70	27.84
Butanol		17.50		17.05		16.16		15.90
Acetone		8.10		8.24		8.42		7.96
Ethanol		2 26		2.08		2.67		3.98
Volatile acid	64	3.17	66	2.49	69	1.48	70	1.43
Formic		0.11		0.07		0.05		0.09
Acetic		2.52		1.80		1.13		1.05
Butyric		0.54		0.62		0.30		0.29
Residual carbohydrate	64	4.91	66	5.69	69	5.83	70	6.66

* This flask had 9.0 cc. of 0.1 N formic acid added per 250 cc. of mash about 5 hours after acidity peak.

TABLE VI.

Percentage Distribution of Volatile Acids.

Concentration of mash.	Time.	Distribution of volatile acids. Formic.	Acetic.	Butyric.
per cent	*hrs.*	*mol per cent*	*mol per cent*	*mol per cent*
3.26	19	0.4	47.4	52.2
3.26	64	5.3	82.6	12.1
5.30	16	0.4	47.6	52.0
5.30	66	4.8	77.0	18.2
7.38	21	0.4	53.1	46.5
7.38	69	5.6	79.9	14.5

3 times that of acetic and butyric, if the former increases, the latter two must decrease.

All cultures contained from 5 to 8 times as much formic acid at the end of the fermentation as at the peak of the acidity (16 to

23 hours). Formic acid is probably the precursor of all of the hydrogen and some of the carbon dioxide. The first part of the fermentation is characterized by a large production of hydrogen and it is at this time that formic acid is at its lowest level.

The solvent production by the various strains is given in Table IV. The use of a different corn-meal of lower carbohydrate content accounts for yields of solvents that were somewhat lower than those obtained in Experiment 2, Table I.

TABLE VII.

Destruction of Added Formic Acid by Clostridium Acetobutylicum.

Determination No.		Experiment 4, Culture A.	Experiment 5, Culture A.
1	Concentration of mash, *per cent*	7.4	7.34
2	Age of culture when formic acid was added, *hrs*	21–27	25–29
3	Acidity of mash at time of first addition, *cc. 0.1 N per 10 cc.*	4.9	3.6
4	Formic acid added, *gm. per liter*	0.236	0.166
5	" " at end of fermentation, *gm. per liter*	0.144	0.068
6	Formic acid in control flask, *gm. per liter*	0.047	0.036
7	Added formic acid unfermented (Determinations 5–6), *gm. per liter*	0.097	0.032
8	Formic acid fermented (Determinations 4–7), *gm. per liter*	0.139	0.134
9	Formic acid fermented, *per cent*	59	81
10	Recovery by distillation of formic acid added (Determinations 4+6), *per cent*	94.8	

The results of varying the strength of the mash with a particular culture (Culture A) are given in Table V. A higher proportion of solvents and volatile acid and a lower percentage of residual carbohydrate were obtained from the lower concentrations of corn.

From the Duclaux data the percentages of the three volatile acids at the acidity peak and at the end of the fermentation have been calculated and are given in Table VI. The dilute mashes developed a higher proportion of butyric acid at the peak of the acidity

(16 to 21 hours) than did the 7 per cent mash, and as these dilute mashes produced more butanol, it is probable that more of the intermediate compound from the starch went through the butyric acid stage. The molar concentration of formic acid remained practically unaffected by changes in the concentration of the mash.

Destruction of Added Formic Acid by Clostridium Acetobutylicum.—If formic acid is an intermediate compound in the formation of

TABLE VIII.

Distribution of Solvents Produced under Various Conditions of Fermentation.

Experiment No.	Culture.	Concentration of dry corn in mash.	Butanol.	Acetone.	Ethanol.
		per cent	*per cent*	*per cent*	*per cent*
1	C	5.4	55.0	32.3	12.7
2	A, Flask 1.	7.4	61.9	33.2	4.9
	" " 2.	7.4	60.3	32.9	6.8
3	" + $CaCO_3$.	6.9	67.1	28.9	4.0
	" control.	7.0	59.8	29.9	10.3
4	"	7.4	61.1	31.0	7.9
	B	7.5	61.9	32.5	5.6
	C	7.5	59.7	30.4	9.9
	D	7.5	57.8	31.5	10.7
	A	7.5	59.0	31.8	9.2
	A*	7.4	58.0	31.4	10.6
5	A	3.3	62.8	29.1	8.1
	"	5.3	62.3	30.1	7.6
	"	7.4	59.3	30.9	9.8
	A†	7.4	57.1	28.6	14.3

* This flask had 12.8 cc. of 0.1 N formic acid added per 250 cc. of mash.

† This flask had 9.0 cc. of 0.1 N formic acid added per 250 cc. of mash.

hydrogen and carbon dioxide, it should be fermentable by the acetone-butyl alcohol organism. To determine this point, formic acid was added to the mash and at the end of the fermentation a determination was made of the unfermented acid. In Experiment 4 the additions were made at 6 hour intervals beginning just after the break in the acidity. Sterile 1 N formic acid was added at the rate of 2.5 cc. per liter of culture. As this quantity of acid depressed the fermentation, the first addition in the next experiment,

No. 5, was deferred a few hours and the quantity was reduced to 1.5 cc. of 1 N acid per liter. The data for the yield of products are given in Tables IV and V and for the destruction of formic acid in Table VII. In Experiment 4 the yield of solvents was reduced to 80.7 per cent of that found in the control flask. In Experiment 5 there was no reduction in yield of solvents but the percentage distribution of the constituents was changed to a marked degree. The effect of added formic acid was to lower the butanol and acetone and to increase the ethyl alcohol. This effect is opposite to that produced by adding calcium carbonate. Data on the distribution of solvents in all the experiments given in Table VIII make this point clearer.

From the data in Table VII it is seen that in Experiment 4 59 per cent and in Experiment 5 81 per cent of the added formic acid was destroyed. Objection may legitimately be made that such a small capacity for destroying added formic acid is not sufficient to explain the destruction of formic acid necessary to account for the hydrogen that is produced. The answer to this objection may be the greater lability of nascent formic acid to decomposition by the organism. Because of its high dissociation in dilute solution formic acid must be decomposed almost as fast as it is formed. If allowed to accumulate, it would soon result in a hydrogen ion concentration detrimental to the bacteria.

Nature of the Non-Volatile Acid.—Schmidt, Peterson, and Fred (5) in their identification of leucic acid as a fermentation product first revealed the nature of at least a portion of the non-volatile acid. Later Speakman (6) obtained positive tests for the hydroxy derivative of tyrosine and suggested that deaminization of amino acids may be a general fermentation reaction. Consequently a method that could be generally applied to α-hydroxy acids was necessary. The permanganate oxidation method of Friedemann and associates (9, 10) for small amounts of lactic acid has been well developed and was found to be applicable to α-hydroxy acids other than lactic. This was demonstrated by the satisfactory recovery of several aldehydes having higher boiling points than acetaldehyde. A Hopkins condenser having a space of 5 mm. between the inner tube and the outer jacket was used in the Friedemann apparatus and the following results were obtained:

Acetaldehyde,	b. p.	21°,	recovery	96.3	per cent.
Butyraldehyde,	"	75–77°,	"	100	" "
Isovaleraldehyde,	"	91–93°,	"	86.6	" "
Benzaldehyde,	"	180°,	"	94.2	" "

These recoveries would indicate that the majority of α-hydroxy acids that arise from deaminization of amino acids occurring in the proteins of corn can be determined by this method.

Quantities of the non-volatile acid and its fractions produced at the various intervals in Experiment 2 are given graphically in Charts 1 and 2. The α-hydroxy acid, as determined by the permanganate oxidation method previously referred to, increases quite rapidly between the 9th and the 12th hour of the fermentation, slowly increases up to the 36th hour, and from then on to the end remains fairly constant.

The *p*-nitrophenylhydrazone of the aldehyde resulting from the oxidation of the α-hydroxy acid was made. The melting point of the crude product was 85-86°. On one recrystallization from ethyl alcohol it melted at 92–93°. It was not identified. Probably a mixture of aldehydes was being dealt with, as presumably several α-hydroxy acids are produced in the fermentation.

The curve for residual non-volatile acid, determined by difference between the total non-volatile and the α-hydroxy acid, as given in Chart 2, showed a definite break, coincident with the sharp break in volatile acidity, and indicated that this residual acid was being converted to neutral products as were the volatile acids. Immediately following the low point in residual acidity there is a second accumulation succeeded by further conversion, and from the 42nd hour on, the amounts of both fractions are quite constant. The α-hydroxy acid, because of the small amount present, is probably all derived from deaminization of amino acids and is a function of protein degradation as Schmidt, Peterson, and Fred (5) and Speakman (6) have previously described. The residual non-volatile acid may be an intermediate product in the conversion of starch to solvents.

SUMMARY.

1. The proportions of formic, acetic, and butyric acids present at progressive time intervals, in the fermentation of corn-meal by *Clostridium acetobutylicum* have been determined. The amount of

formic acid is small during the first 30 hours, but as the total acidity recedes, as much as 0.13 cc. of 0.1 N acid per 10 cc. may be present. In the early stages of the fermentation, the amounts of acetic and butyric acids are nearly equal. Near the peak of acidity concentration butyric acid is slightly in excess. In the latter part of the fermentation the quantity of acetic acid exceeds that of butyric. The amount of butyric acid remaining at the end is found to bear a relationship to the amount of butanol produced.

2. Four different cultures and one culture in three concentrations of corn mash were found to contain small amounts of formic acid.

3. Destruction of added formic acid was obtained. This gives weight to the hypothesis that formic acid is the precursor of the H_2 and some of the CO_2 which are evolved in the fermentation.

4. By the use of $CaCO_3$ about 40 per cent of the intermediate acids were retained as the calcium salts. Analysis by the Fincke and Duclaux methods showed the presence of 0.4 per cent formic, 42.1 per cent acetic, and 57.5 per cent butyric acid by weight. The presence of a fourth volatile acid was indicated by the Duclaux data.

5. The non-volatile acid produced in the fermentation has been determined at frequent intervals, and has been separated into an α-hydroxy acid and a residual fraction. The latter gives evidence of being partially destroyed at the time of receding volatile acidity. The α-hydroxy portion accounted for approximately 20 per cent of the total non-volatile acid at the beginning and increased to about 35 per cent at the end of the fermentation. Data obtained on the melting point of the *p*-nitrophenylhydrazone, made through oxidation of the α-hydroxy acid to the corresponding aldehyde, indicated a mixture of aldehydes and consequently a number of α-hydroxy acids in that fraction of the non-volatile acid.

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STUDIES ON THE ISOMERIZATION OF ERGOSTEROL.

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Isomerization with Hydrochloric Acid.

According to Reindel, Walter, and Rauch (1) hydrogen chloride converts ergosterol into the modification, isoergosterol. In connection with a recent investigation on vitamin D (2) we attempted to prepare isoergosterol by the published method, but we obtained a product markedly different from the one described.

The procedure employed by the above authors and by us consisted in passing dry hydrogen chloride through a chloroform solution of ergosteryl acetate at the temperature of ice water for 1 hour. The chloroform was evaporated, and the isoergosteryl acetate recovered and saponified to the free sterol. The original isoergosterol was said to melt at 136°; $[\alpha]_D = -87°$ in $CHCl_3$. Our product melted at 138°; $[\alpha]_D^{25} = -40°$.

Further work showed that the reaction is best conducted by shaking a chloroform solution of ergosterol with concentrated hydrochloric acid. On addition of the acid a gelatinous white precipitate is formed, which slowly disappears, giving a light green solution.

2 gm. of purified ergosterol (Ergosterol A-B; see below) were dissolved in 70 cc. of chloroform. 4.0 cc. of HCl, sp. gr. 1.18, were added, and the mixture was transferred to a shaking machine in a constant temperature dark room. The shaking was continued for 4 hours at 25° ± 2°. The chloroform layer was separated from the aqueous layer and rapidly evaporated over a boiling water bath in the dark. The residue was comminuted with 15 cc. of 96 per cent alcohol, refrigerated, filtered with suction, washed with four 5 cc. portions of ice-cold alcohol, and dissolved in 40 cc. of hot alcohol. The solution was crystallized,

refrigerated, filtered, and recrystallized once from 30 cc. of alcohol. The product was dehydrated in a high vacuum at 80° for 10 minutes.

This isomerization was repeated several times, all factors being exactly as described, except the duration of treatment. By observing the specific rotation after different periods, we were able to construct a curve (Fig. 1) which depicts the course of isomerization. From Fig. 1 it is evident that isomerization

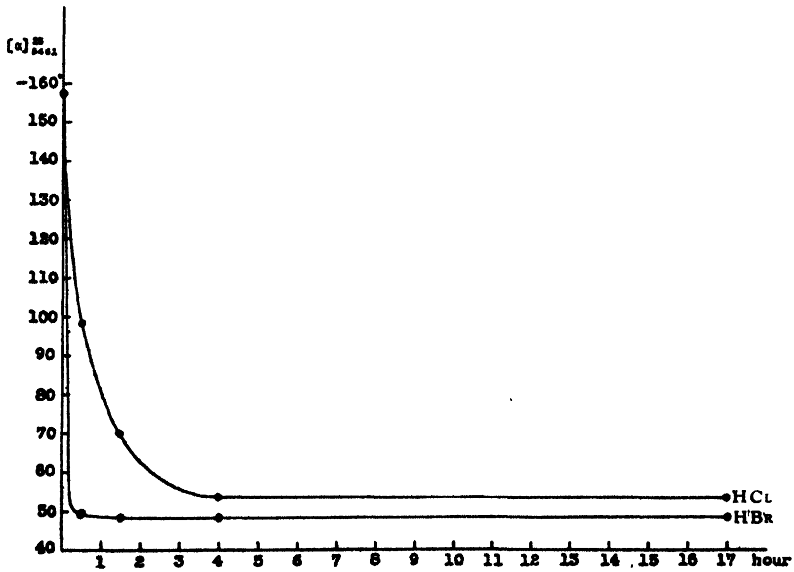

FIG. 1. Isomerization of ergosterol with HCl and HBr.

became complete in about 4 hours, and that further treatment, up to 17 hours, effected no change. Fig. 1 also indicates that the isoergosterol originally described by Reindel, Walter, and Rauch was an incompletely isomerized product.

Isomerization with Hydrobromic Acid.

Since the reactivity of halogen acids with an organic compound generally increases with their molecular weight, we considered it worth while to use hydrobromic acid in an attempt to speed the

isomerization. A second series of treatments was made with the same sample of ergosterol, every detail of the experiment being as described above except that hydrobromic acid, sp. gr. 1.39, was employed instead of hydrochloric acid. Only a small amount of the gelatinous substance was formed, and this quickly disappeared, giving an emerald-green solution. There was very little decomposition—no more, apparently, than with hydrochloric acid.

The curve of isomerization with hydrobromic acid is also shown in Fig. 1. It reveals not only that complete isomerization was attained within 1 hour, but that *the isomer produced was different from the isomer with hydrochloric acid.* The hydrobromic isomer gave $[\alpha]_{5461}^{25} = -48.5°$ in $CHCl_3$ ($c = 1$), while the hydrochloric isomer gave $[\alpha]_{54}^{25} = -53.5°$. Fuming hydrobromic acid, sp. gr. 1.49, yielded an isomer identical with the one produced by acid of ordinary concentration. This difference in specific rotation, 5°, was much greater than the experimental error, for in repeated tests we duplicated results to within 0.1° or 0.2°.

Early in our experimentation we considered that isoergosterol was one distinct substance, and when we first obtained the hydrobromic isomer we suspected that the hydrochloric isomer was an imperfectly isomerized form. That this is not the case is demonstrated by the remarkable fact that *the isomers are intertransformable.*

Isomerization with Cinnamoyl Chloride.

A year ago Bills and Honeywell (3) described an ester, presumed to be ergosteryl cinnamate, which was produced by melting together ergosterol and cinnamoyl chloride at 170°. Recently Windaus and Rygh (4) reported that the free sterol obtained by saponifying this ester was not ergosterol. They argued, moreover, that it was not isoergosterol, because it did not answer the description of Reindel's product. The Windaus and Rygh sterol gave $[\alpha]_D^{17} = -35°$, a value which suggests close relationship to our forms of isoergosterol.

To investigate further the cinnamic isomer we prepared a new supply of the sterol cinnamate, exactly following the procedure of Bills and Honeywell, except that in this instance we used the

same batch of purified ergosterol that was used for the hydrochloric and hydrobromic isomers. This ergosterol, as we shall explain below, differed from the ergosterol employed last year.

10 gm. of the new isoergosteryl cinnamate were refluxed with 500 cc. of 2 per cent KOH in 96 per cent alcohol. Boiling was continued for 5 minutes after the last crystal dissolved, and then to the hot solution 125 cc. of hot water were added. The product was refrigerated, filtered with suction, washed with 65 per cent alcohol, and then with 50 per cent alcohol until the filtrate was neutral to litmus. The fine, colorless crystals were recrystallized twice from alcohol. Yield, 5.7 gm. of leaflets with characteristic luster, indistinguishable by appearance from the hydrochloric and hydrobromic isomers. The foregoing saponification technique is generally applicable to sterol esters, and is preferable to the more severe treatments commonly employed.

The sterol prepared via the cinnamate gave $[\alpha]_{5461}^{25} = -39.2°$ when $c = 1$ in $CHCl_3$. Its rotation was thus 9.3° less than that of the hydrobromic isomer, and 14.3° less than the hydrochloric. It was therefore a third form of isoergosterol.

The three isomers, singly or admixed, melted clear, without yellowing, in the neighborhood of 140°. The clearing point was preceded by a sintering range of about 5°. The slight differences which were observed in the melting point of each isomer were not determined with sufficient accuracy to report. The melting point of isoergosterol (HBr), unlike that of ergosterol (3), did not vary greatly with the moisture content of the sample.

Intertransformation of Isomers.

It is demonstrated in Fig. 1 that the reduction in specific rotation ceases within a short time, and that prolongation of the treatment effects no further change. Furthermore, we have found that neither the hydrochloric nor the hydrobromic isomer suffers any reduction in rotation when retreated with the acid which produced it.

However, when the purified hydrochloric isomer was treated for 2 hours in the usual manner with hydrobromic acid, the specific rotation of the hydrobromic isomer was obtained, $[\alpha]_{5461}^{25} = -48.8°$. When the purified hydrobromic isomer was treated with cinnamoyl chloride as described above, the specific rotation

was further reduced to that of the cinnamic isomer, $[\alpha]_{5461}^{25} = -39.7°$.

The reverse transformations were even more remarkable. The purified cinnamic isomer upon treatment with HBr gave the hydrobromic isomer, $[\alpha]_{5461}^{25} = -49.2°$; and with HCl the hydrochloric isomer, $[\alpha]_{5461}^{25} = -54.4°$. It will be noted that in all these cases of retreatment, the observed specific rotation was slightly greater than the expected value (Table I).

TABLE I.

Showing the Specific Rotation of Different Isoergosterols.*

$[\alpha]_{5461}^{25}$; $c = 1$ in $CHCl_3$.

Isomer.	Original specific rotation.	Specific rotation after retreatment with:		
		Hydrochloric acid.	Hydrobromic acid.	Cinnamoyl chloride.
	degrees	*degrees*	*degrees*	*degrees*
Hydrochloric acid, A-B	−53.5	−54 9	−48.8	
" " F	−56.6			
Hydrobromic " A-B	−48.5	−55 2	−49 4	−39 7
" " F	−50.0			
Cinnamoyl chloride, A-B	−39 2	−54 4	−49 2	
" " F	−40 3	−56 3		

* The rotation with sodium light may be calculated, $[\alpha]_{D}^{25} = [\alpha]_{5461}^{25} \div 1.27$. This relation was established for the isomer prepared with HBr; it holds also for ergosterol itself and presumably for the other isomers.

We have already mentioned that in the treatment of ergosterol with HCl and HBr the chloroform solution acquired a green color and there was slight decomposition. It is noteworthy that in treatment of the hydrochloric, hydrobromic, and cinnamic isomers with either of these acids, the green color was absent, and the decomposition negligible. This would indicate either that the isomers were more stable than the original ergosterol (yet readily intertransformable), or that the ergosterol contained a small amount of a highly unstable chromogenic contaminant.

By exposing an oxygenated alcoholic solution of ergosterol and

eosin to visible light, we formed the ergosterol peroxide of Windaus and Brunken (5). Under the same conditions no peroxide was formed from isoergosterol (HCl). Since dihydroergosterol likewise does not form a peroxide (5), and neither dihydroergosterol nor isoergosterol is antiricketically activatable (5, 6), we would tentatively suggest that *the double bond which is saturated in dihydroergosterol is the one that is shifted in isoergosterol, and "activated" in vitamin D.*

Evidence of Natural Isomers.

There is evidence that in addition to the artificially prepared isomers, natural isomeric forms of ergosterol exist. The ergosterol described by Bills and Honeywell exhibited $[\alpha]_{5461}^{25} = -165.6°$. It had been freed from zymosterol and cerevisterol by two recrystallizations from alcohol-benzene (2:1), and additional recrystallizations had effected no change in its specific rotation. This ergosterol was prepared from a commercial bakers' yeast—a strain of *Saccharomyces cerevisiæ* grown in an aerated molasses-ammonia medium (Yeast F, Ergosterol F). As Yeast F gave low yields of ergosterol, we changed to Yeast A-B, a different strain of *Saccharomyces cerevisiæ*, grown in aerated cereal wort, and yielding 10 times more sterol.

300 gm. of crude Ergosterol A-B were recrystallized five times from alcohol-benzene (2:1). With each treatment there was a small increase in levorotation, the fifth product showing 0.9° more than the fourth. (Ergosterol F under identical conditions became constant with two recrystallizations.) Since it appeared that many more crystallizations would be necessary before constancy could be expected, we stopped after the fifth treatment. The yield was 120 gm., exhibiting $[\alpha]_{5461}^{25} = -156.9°$. This ergosterol, rotating 8.7° less than the highly levorotatory Ergosterol F, was the material with which the foregoing experiments on isomerization were made. The fact that its specific rotation was still on the increase may account for the phenomenon that in the experiments on retreatment of isomers with acid, the final products always showed slightly greater levorotation than the expected values.

Ergosterol A-B and Ergosterol F were both highly activatable, beautifully crystalline, and undoubtedly free from zymosterol and

cerevisterol. A possible explanation of the difference in their optical activity is afforded by a comparison of the isomers prepared from each. The isomer obtained from Ergosterol F by treatment with HCl gave $[\alpha]_{5461}^{25} = -56.6°$, or 3.1° greater than the corresponding isomer of Ergosterol A-B. Similarly, the hydrobromic and cinnamic isomers of Ergosterol F showed 1.5° and 1.1° higher specific rotation than the corresponding isomers of Ergosterol A-B.

The rotatory difference in the two original ergosterols became progressively less with the three isomers, until in the cinnamic isomer the difference was only 1.1°. This might be explained by the hypothesis that Ergosterol F contained a strongly levorotatory natural isomeric contaminant difficult to isomerize. As the isomerizing treatment became increasingly severe, this contaminant was more and more changed. On the other hand, the fact that retreatment of the isomers (Ergosterol A-B) with acid always gave a slightly higher rotation than was obtained on initial treatment with acid, but a rotation which never equalled that of the corresponding Ergosterol F isomers, suggests that Ergosterol A-B contained a natural isomeric form of lower specific rotation, from which the isomers were partially freed by the extra crystallizations involved.

Whichever be the correct explanation, we are of the opinion that the natural isomeric contaminant is characterized by isomerism in a different part of the molecule than the double bond concerned in the formation of the artificial isoergosterols. If one of our isoergosterols were the natural contaminant of Ergosterol F or Ergosterol A-B, then these ergosterols upon treatment with acid should yield identical isoergosterols, but they do not. Although the cinnamic isomers differed in specific rotation by only 1.1°, they were readily transformed by HCl into the respective hydrochloric isomers. Like all other Ergosterol A-B isomers subjected to retreatment, the hydrochloric isomer prepared from the cinnamic isomer exhibited a slightly increased optical activity (0.9°). Yet the hydrochloric isomer prepared from the cinnamic isomer of Ergosterol F showed almost the same specific rotation (actually 0.3° less) than did the hydrochloric isomer obtained directly from Ergosterol F (Table I). Thus we see that the natural difference between Ergosterol F and Ergosterol A-B persists in the three isomers, even after intertransformation.

Recently Heilbron, Sexton, and Spring (7) investigated two naturally different ergosterols somewhat similar to our specimens, Ergosterols F and A-B. They found that the form which had the higher levorotation absorbed hydrogen less readily; also that it was apparently converted into the other form via the acetate. In connection with the latter observation it is interesting that our *less* levorotatory form (Ergosterol A-B), when heated with monochloroacetic acid, yielded the chloroacetate of the hydrobromic isomer.

2 gm. each of Ergosterol A-B and chloroacetic acid were heated at 170° for 10 minutes. The melt became emerald-green, turning to olive. The product was washed with water and dilute acetone; it was crystallized once from acetone and once from alcohol-benzene (2:1). Yield, 1 gm. of snow-white leaflets, very insoluble in alcohol; m.p. 196°; $[\alpha]_{5461}^{25} = -62.6°$ when $c = 0.5$ in $CHCl_3$. Saponification gave isoergosterol $[\alpha]_{5461}^{25} = -48.7°$.

SUMMARY.

1. Three forms of isoergosterol result from treatment of ergosterol with (1) hydrochloric acid, (2) hydrobromic or chloroacetic acid, and (3) cinnamoyl chloride.

2. The three isomers are intertransformable, any one changing into another upon treatment with the appropriate acid.

3. This isomerism appears to involve the same double bond which is saturated in dihydroergosterol and "activated" in vitamin D.

4. Ergosterol prepared from different cultures of yeast may exhibit a natural isomerism involving some part of the molecule other than the double bond affected by acid.

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THE UTILIZATION OF THE CALCIUM OF CALCIUM CARBONATE AND CITRATE BY LAYING AND NON-LAYING PULLETS.*

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The results of common practice and feeding experiments teach that calcium in some concentrated form must be supplied in the ordinary grain mix fed poultry in order to correct the deficiency of this element and to supply the large amount necessary for optimum egg production. For instance, Buckner and Martin (1) have demonstrated that a deficient calcium supply results in a thinning of the egg shell, and a decrease and final cessation of egg production. Furthermore, poor hatchability of hen's eggs is reported by Buckner and coworkers (2) when the calcium supply is inadequate.

Little attention has been given to the question of the availability of calcium from different compounds for egg shell formation. In 1922 Buckner and his associates (3) reported that it would seem that the hen can utilize calcium carbonate for both egg shell and bone formation, but that the calcium from tricalcium phosphate can be utilized only for bone growth and not for egg shell formation. Later (1928) the same investigator (4) and his

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associates, in a study of the comparative effectiveness of calcium carbonate, calcium sulfate, calcium lactate, precipitated tricalcium phosphate, and calcium chloride in the production of eggs, demonstrated that the carbonate is the most effective source. Increases in production were obtained in the case of the other compounds when they were fed after a period of exclusion of calcium from the ration, the smallest being obtained with the phosphate and chloride. The last named compound was not well tolerated.

The primary object of the present investigation was to ascertain whether or not the laying pullet can utilize the calcium from calcium citrate for egg production and shell formation, and if so, which may be considered the better source of calcium, calcium citrate or carbonate. Since information of a quantitative nature was desired, it was decided to use the balance method. Halnan (5) employed this method in a study in which calcium carbonate was the source of calcium. He demonstrated that calcium was not stored to any great extent for a long time preceding egg production, but that the demands of the birds were almost completely met by the increased retention of calcium from the available food supply, during the egg-producing period. Negative calcium balances were recorded during periods of heavy production. Halnan, however, did not compare the availability of calcium in the form of the carbonate with that of the element in other compounds. In addition to the calcium balance he determined that for phosphorus, and likewise in the present study the phosphorus balance was determined in order to learn something of the rôle of this element in the economy of the chicken and of its relation to the calcium data.

Experimental Procedure.

Pullets were used instead of hens in order to reduce the possibility of disturbance of egg laying by change of environment from one of relatively large freedom to close confinement. It was considered probable that the younger bird which had not started egg production would be more adaptable to close confinement in a metabolism cage.

Eight White Leghorn pullets just about to begin egg production were used. After 7 weeks of preliminary feeding calcium and phosphorus balance determinations were started August 5, 1927,

and conducted for 5 weeks. The same ration, described below, was used in the preliminary and experimental periods. The birds were kept in individual compartments of a fattening battery[1] which served as a metabolism cage. The floor area of each compartment was 17″ × 30″. Each cage was fitted below the wire screen bottom with a sliding metal tray which was turned up about ¼ inch on all four sides. The feed containers were constructed especially to reduce the mechanical loss of food due to scattering. Even with the utmost care it was impossible to prevent a slight loss of food, but it is estimated that the loss was too small to affect appreciably the total calcium intake per week. The droppings were collected twice weekly and dried in a porcelain dish for 1 week in an oven at 65°. They were then ground to a fine powder and sampled for analysis. Each week the trays were replaced with clean ones. Any feathers shed during the experiment were collected and discarded. Halnan (5) found the largest amount of calcium and of phosphorus in a week's collection of feathers to be 0.02 gm. The basal ration consisted of a mixture of ground whole yellow corn 61 per cent, ground whole hard wheat 24 per cent, casein 12 per cent, sodium chloride 1 per cent, and cod liver oil 2 per cent. To this basal ration there was introduced at the expense of the corn a calcium salt in sufficient quantity to bring the calcium content of the diet to 1.4 per cent as shown by analysis.[2] The phosphorus content was 0.34 per cent. Four pullets, Nos. 5 to 8 received calcium citrate as the source of calcium, while Pullets 9 to 12 received calcium carbonate. The ration was well mixed and supplied *ad libitum* as a whole, dry mash. Distilled water was available at all times.

The eggs were collected as soon after being laid as possible in order to prevent breakage by the pullet as it moved about the cage. They were weighed at the time of collection. The shells and contents were analyzed separately. The eggs were broken, the contents separated from the shells, and any adhering contents washed free with distilled water. The shell membrane was analyzed

[1] A Wonder fattening battery made by the Collis Company, Clinton, Iowa, was used.

[2] The calcium citrate was supplied by Chas. Pfizer and Company, Inc. The calcium carbonate, precipitated, Tested Purity grade, was obtained from Eimer and Amend, New York.

with the shell. Distilled water was added to the egg contents. and the whole well stirred with an egg beater. The foam which resulted from the stirring was broken by the addition of a small amount of ether and the mixture diluted to a known volume. An aliquot of this solution was taken and carried to dryness at 65°. The resulting dry material was ashed for calcium and phosphorus determinations. The shells and shell membrane, washed free from the egg contents, were dried at 65°, weighed, and ashed. They were then dissolved, diluted to a known volume, and calcium and phosphorus determinations made of aliquots. Calcium was determined by the method of McCrudden (6). Phosphorus was determined by the volumetric method (7).

TABLE I.

Initial Weight of Birds and Gain in Weight.

Pullet No.	Weight at beginning of preliminary feeding.	Gain in weight over entire period.
	gm.	*gm.*
5	1105	257
6	1275	341
7	1219	153
8	1049	255
9	1162	29
10	1219	85
11	1219	256
12	1275	370

General Health of Birds.

The condition of the birds' health remained good throughout the entire period of confinement as far as one could observe from the action and general contentment of the individuals. They seemed to be satisfied and did not appear nervous. The weights of the birds at the beginning of the preliminary feeding and the gains in weight during the entire period of confinement are shown in Table I.

Although there was no loss in weight, the gain was very slight in some cases and in most cases not what would have probably been made if more freedom had been allowed. The number of individuals was too small to permit any conclusions relative to differences in growth as induced by the two calcium salts.

TABLE II.

Record of Calcium Balances of Pullets Receiving Calcium Citrate.

Week.	Food consumption.	Weight of dry droppings.	No. of eggs produced.	Weight of eggs produced.	Weight of shell.	Per cent of shell by weight of total eggs.	Ca in shells.	Per cent of shell Ca by weight of total eggs.	Ca in egg contents.	Ca in droppings.	Total Ca intake.	Total Ca output.	Ca balance per wk
	gm.	gm.		gm.	gm.		gm.		gm.	gm.	gm.	gm.	gm.
Pullet 5.													
1	403	80 8	3	122.07	11.60	9.0	4.15	3.39	0 07	1.73	5.64	5.95	−0.31
2	441	84.7	4	174.73	15.56	9.9	5.51	3.15	0.12	1.17	6.17	6.80	−0.63
3	373	72.0	3	132.58	11.35	8.5	4.01	3.02	0.07	0.83	5.22	4.91	+0.31
4	348	65.2	3	126.43	10.22	8.0	3.73	2.95	0.09	1.14	4.87	4.96	−0.09
5	400	84 4								4.12	5.60	4.12	+1.48
Pullet 6.													
1	326	63.5								2.48	4.56	2.48	+2.08
2	356	77.5								3.43	4.98	3.43	+1.55
3	436	73.1								3.92	6.10	3.92	+2.18
4	346	55 8	1	44 11	3 66	8 2	1 34	3.04	0.04	2.40	4.84	3.78	+1.06
5	336	64 9								4.39	4.70	4.39	+0.31
Pullet 7.*													
1	194	72.2	3							2.56	2.72	2.56	+0.16
2	292	66 6								2.78	4.09	2.78	+1.31
3	272	74 0	2							3.04	3.81	3.04	+0.77
4	251	63.3	1							2.71	3.51	2.71	+0.80
5	370	79.7								3 01	5.18	3.01	+2.17
Pullet 8.													
1	333	74.4								3.22	4.66	3.22	+1.44
2	336	73.3								3.21	4.70	3.21	+1.49
3	430	83.5								4.99	6.02	4.99	+1.03
4	333	74 9								4.13	4.66	4.13	+0.53
5	313	69 0								4.36	4.38	4.36	+0.02

During the preliminary feeding the birds produced the following numbers of eggs: Pullet 5, 4; Pullet 6, 10; Pullet 7, 11; Pullet 8, 11.

*Pullet 7 developed into an egg eater and this record is not considered from the quantitative standpoint. Qualitatively it is comparable with the records of other individuals.

TABLE III.

Record of Calcium Balances of Pullets Receiving Calcium Carbonate.

Week.	Food consumption.	Weight of dry droppings.	No. of eggs produced.	Weight of eggs produced.	Weight of shell.	Per cent of shell by weight of total egg.	Ca in shells.	Per cent of shell Ca by weight of total egg.	Ca in egg contents.	Ca in droppings.	Total Ca intake.	Total Ca output.	Ca balance per wk.
	gm.	*gm.*		*gm.*	*gm.*		*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Pullet 9.													
1	349	72.6	2	84.78	8.23	9.7	2.82	3.32	0.05	1.97	4.89	4.84	+0.05
2	262	57.0	4	162.19	14.47	8.8	4.87	3.00	0.11	0 88	3.67	5.86	−2.19
3	311	62.4	2	77.59	7.30	9.4	2.71	3.48	0.04	1.16	4.35	3.91	+0.44
4	247	54.7	2	82.54	6.67	8.0	2.40	2.90	0.07	1.14	3.46	3.61	−0.15
5	300	69.0								3.70	4.20	3.70	+0.50
Pullet 10.													
1	207	52.2	1	35.67	3.27	9.1	1.19	3.32	0.03	0.77	2.90	1.99	+0.91
2	219	51.6								1.74	3.08	1.74	+1.34
3	316	76.7								2.78	4.42	2.78	+1.64
4	337	71.9								3.64	4.72	3.64	+1.08
5	289	66.8								4.30	4.05	4.30	−0.25
Pullet 11.													
1	249	57.1								1.87	3.49	1.87	+1.62
2	238	54.3								1.78	3.33	1.78	+1.55
3	375	74.2								3.96	5.25	3.96	+1.29
4	370	69.3								3.28	5.18	3.28	+1.90
5	389	81.0								4.08	5.45	4.08	+1.37
Pullet 12.													
1	435	76.1	3	122.07	11.89	9.7	4.21	3.44	0.08	1.34	6.09	5.63	+0.46
2	455	88.6	3	126.10	12.26	9.7	4.39	3.48	0.10	2.36	6.37	6.85	−0.48
3	411	79.1	1	47.03	4.53	9.6	1.65	3.51	0.02	3.04	5.75	4.71	+1.04
4	417	89.1								6.43	5.84	6.43	−0.59
5	358	89.2								5.41	5.01	5.41	−0.40

During the preliminary feeding the birds produced the following numbers of eggs: Pullet 9, 6; Pullet 10, 20; Pullet 11, 16; Pullet 12, 6.

Results of Calcium Balance Study.

Tables II and III give the detailed experimental data and the calcium balances obtained during production and non-production. The food consumption is lower than that observed in birds allowed

greater freedom. The smaller food consumption, and the consequent slight gain in some cases, is probably due to the close confinement under which the birds were maintained.

The average weight of the eggs was about 41 gm., which is somewhat less than that observed in the case of pullets coming into production under more normal conditions, the average in the latter case being closer to 50 gm. The failure to produce more eggs and eggs of greater weight may have been due in part to the calcium supply. The calcium in the ration was adjusted at a level which was estimated to be less than optimum, so that any differences between the utilization of the two sources might be accentuated.

In both groups the calcium balances approach the negative side and there is a lowered excretion of calcium in the droppings during periods of production, which is excellent evidence that calcium from both sources is available for egg-shell formation. With two exceptions (certain weeks of the records of Pullets 10 and 12) the balances of both groups became more positive during non-production, which indicates a retention for bodily needs, and there is a higher excretion in the droppings.

The data are not displayed but the breaking strength and grades of eggs produced by each group are equal in these respects. Furthermore, egg weights and the percentage of calcium in the shell were very similar on the basis of the weights of the whole egg, all of which is added evidence that calcium is equally available from the two sources.

Results of Phosphorus Balance Study.

Tables IV and V give the experimental data concerning the phosphorus balances and fluctuations in the phosphorus excreted. During the experimental period all birds showed phosphorus retention except Pullets 5 and 9, whose records indicate negative balances in the 2nd and 3rd weeks. Although the negative balances are not marked, except in the case of Pullet 9, 2nd week, they suggest that with high production there may be periods of negative phosphorus balance. On the other hand Pullet 12 was in positive balance during the 2nd and 3rd weeks while in egg production. The data are not sufficient to allow the drawing of conclusions to account for this difference in behavior but it should be

TABLE IV.

Record of Phosphorus Balance of Pullets Receiving Calcium Citrate.

Week.	Food consumption.	Weight of dry droppings.	No. of eggs produced.	Weight of eggs produced.	P in shell.	P in egg contents.	P in droppings.	Total P intake.	Total P output.	P balance per wk.	Calculated amount of P to form $Ca_3(PO_4)_2$ with excretory Ca.	Ratio of Ca:P in droppings.
	gm.	*gm.*		*gm.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Pullet 5.												
1	403	80.8	3	122.07			0.97	1.33		*	0.89	1.7
2	441	84.7	4	174.73	23.1	273.7	1.41	1.46	1.71	−0.25	0.60	0.8
3	373	72.0	3	132.58	17.8	208.5	1.22	1.23	1.45	−0.22	0.42	0.6
4	348	65.2	3	126.43	15.5	190.7	0.71	1.15	0.92	+0.23	0.58	1.6
5	400	84.4					0.75	1 32	0.75	+0.57	3.12	5 5
Pullet 6.												
1	326	63.5					0.14	1.08	0.14	+0.94	1.28	17.7
2	356	77.5					0.33	1.18	0.33	+0.85	1.77	10.4
3	436	73.1					0.37	1.44	0 37	+1.07	2.02	10.6
4	346	55.8	1	44.11	8.0	70.4	0.37	1.14	0.45	+0.69	1.24	6.4
5	336	64.9					0.93	1.11	0.93	+0.18	2.26	4.7
Pullet 7.†												
1	194	72.2	3				0.51	0.64		*	1.32	5.0
2	292	66.6					0.13	0.96	0.13	+0.83	1.43	21.3
3	272	74.0	2				0.70	0.90			1.57	4.3
4	251	63.3	1				0.19	0.83			1.37	14.2
5	370	79.7					0.18	1.22	0.18	+1.05	1.55	16.7
Pullet 8.												
1	333	74.4					0.24	1.10	0.24	+0.86	1.66	13.4
2	336	73.3					0.35	1.11	0.35	+0.76	1.65	9.1
3	430	83.5					0.84	1.42	0.84	+0.58	2.74	5.9
4	333	74.9					0.63	1.10	0.63	+0.47	2.13	6.5
5	313	69.0					0.79	1.03	0.79	+0.24	2.25	5.5

* Phosphorus determinations were not carried out on the egg contents and shells during the 1st week.

† Pullet 7 developed into an egg eater and this record is not considered from the quantitative standpoint. Qualitatively it is comparable with the records of other individuals.

noted that the number of eggs produced by Pullet 12 was not as large as in the cases of Pullets 5 and 9. The tendency toward a

TABLE V.

Record of Phosphorus Balance of Pullets Receiving Calcium Carbonate.

Week.	Food consumption.	Weight of dry droppings.	No. of eggs produced.	Weight of eggs produced.	P in shell.	P in egg contents.	P in droppings.	Total P intake.	Total P output.	P balance per wk.	Calculated amount of P to form $Ca_3(PO_4)_2$ with excretory Ca.	Ratio of Ca:P in droppings.
	gm.	*gm.*		*gm.*	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Pullet 9.												
1	349	72.6	2	84.73			1.23	1.15		*	1.01	1.6
2	262	57.0	4	162.19	15.7	255.8	1.41	0.87	1.69	−0.82	0.45	0.6
3	311	62.4	2	77.59	11.3	120.5	1.01	1.03	1.14	−0.11	0.59	1.1
4	247	54.7	2	82 54	11.3	133.3	0.57	0.82	0.71	+0.11	0.58	2.0
5	300	69.0					0.72	0.99	0.72	+0.27	1.91	5.1
Pullet 10.												
1	207	52.2	1	35.67			0.22	0.68		*	0.39	3.4
2	219	51.6					0.09	0.72	0.09	+0.63	0.89	19.3
3	316	67.7					0.16	1.04	0.16	+0.88	1.43	17.3
4	337	71.9					0.43	1.11	0.43	+0.68	1.87	8.4
5	289	66.8					0.89	0.95	0.89	+0.06	2.38	4.8
Pullet 11.												
1	249	57.1					0.09	0.82	0.09	+0.73	0.96	20.7
2	238	54.3					0.07	0.79	0.07	+0.72	0.92	25.4
3	375	74.2					0.33	1.24	0.33	+0.91	2.04	12.0
4	370	69.3					0.20	1.22	0.20	+1.02	1.69	16.4
5	389	81.0					0.41	1.28	0.41	+0.87	2.10	9.9
Pullet 12.												
1	435	76.1	3	122.07			1.31	1.44		*	0.69	1.0
2	455	88.6	3	126.10	18.0	196.1	0.70	1.50	0.91	+0.59	1.21	3.3
3	411	79.1	1	47.03	6.0	66.2	0.71	1.36	0.78	+0.58	1.56	4.2
4	417	89.1					1.28	1.38	1.28	+0.10	3.32	5.0
5	358	89.2					1.05	1.18	1.05	+0.13	2.79	5.1

* Phosphorus determinations were not carried out on the egg contents and shells during the 1st week.

negative phosphorus balance during production emphasizes the need of more attention to the phosphorus requirements of the chicken.

Although the weight of phosphorus retained per week is not as great as that of the calcium, the percentage of the phosphorus intake retained during non-production is about twice that of the calcium. In some instances the percentage retention of phosphorus was as high as 80 to 90 per cent. It is of further interest that more phosphorus was excreted per week in the droppings by Pullets 5 and 9 during production in the 1st, 2nd, and 3rd weeks than in the last week of production (the 4th) and the 5th week, when no eggs were produced. In the case of Pullet 12 the excretion in the droppings during the 1st week when three eggs were produced was slightly greater than in the 4th or 5th when there were no eggs produced. During production in the 2nd and 3rd weeks, however, less phosphorus was excreted than during non-production. It is also observed by Halnan (5) that as laying begins considerably more phosphorus is excreted than is necessary for the excretory calcium requirement and he states that "it would appear that this extra P_2O_5 excretion is necessarily linked with the metabolic changes involved in egg production". Although our records do not cover as long a period as those of Halnan, yet they are in general agreement in that more phosphorus was excreted during egg production in the majority of cases than was necessary for the formation of $Ca_3(PO_4)_2$ with the excretory calcium and they likewise suggest a metabolic change involving phosphorus compounds, during production (Tables IV and V).

Halnan (5) points out that the calculated amount of P_2O_5 required to form $Ca_3(PO_4)_2$ with the CaO excreted, if one assumes that phosphorus is excreted in this form, is in good agreement with the actual phosphorus excreted, when the bird is not producing eggs. If one computes from the data of Halnan the actual phosphorus instead of the P_2O_5, it is found that the agreement is still very good, as would be anticipated, between the actual and theoretical amounts excreted. The data when calculated from the results obtained in this experiment, however, do not support the above contention. The records in Tables IV and V show that the phosphorus excreted by the non-laying pullets is far less than that required by theory. This might be accounted for in that the phosphorus intake was much lower in this experiment

than that reported by Halnan. The phosphorus relationships are apparently the same in the case of both sources of calcium.

Calcium-Phosphorus Ratio.

If the calcium-phosphorus ratio in the droppings of both laying and non-laying pullets is calculated, the relationship between the two elements, pointed out above, is shown in a more striking fashion. These two ratios have been placed in Tables IV and V. It is observed that the ratio is generally quite low during egg production and, on cessation of laying, that the ratio increases and remains higher in the non-laying individuals. Our results confirm those of Halnan (5) which show the same relationship when calculated in the same manner.

SUMMARY.

1. Negative or very slightly positive balances and a lowered excretion of calcium in the droppings during egg production are excellent evidence that the laying pullet can utilize calcium from calcium citrate as well as from the carbonate for egg formation. Positive balances during non-production indicate a retention for bodily needs.

2. The breaking strength, grades, egg weights, and percentage of calcium in the shell were equal in these respects for both sources of calcium.

3. For both groups of birds the data suggest that with high production there may be periods of negative phosphorus balance. In general during production more phosphorus is excreted in the droppings than during non-production and more than is necessary to form tricalcium phosphate with the excretory calcium. These observations suggest a metabolic change involving phosphorus compounds during egg production.

4. During non-production the percentage of phosphorus retained is greater than that of the calcium and the phosphorus in the droppings is less than that required for the formation of tricalcium phosphate.

5. The ratios of calcium to phosphorus in the droppings of laying and non-laying pullets show a lower ratio during the period of egg production.

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THE ACTION OF SAPONIN ON ANTITOXIN.*

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For some years, the possibility of separating proteins, particularly those possessing similar physical properties, by means of various detergents, has been of interest to us. In 1925, it was shown by one of us (D.A.S.) that when trypsin was added to insulin, the biological action of the insulin was immediately and completely inhibited. Many experiments were carried out in an attempt to liberate the combined insulin from the insulin-trypsin complex by various detergents, such as saponin, sodium taurocholate, and sodium glycocholate. These experiments were unsuccessful in liberating the insulin (1). Following this, many experiments were carried out in an attempt to fractionate the proteins in antitoxin by means of the detergent action of saponin. We were not successful in effecting a purification, but observed that saponin would precipitate a great part of the protein at an acidity of pH 4. More recently, Harington and Scott (2) showed that insulin could be freed from amorphous material, in such a way that it would separate out in crystalline form by the use of an active saponin. These results stimulated us to reinvestigate the action of saponin on antitoxin. Particularly did this seem desirable in view of the fact that Harington and Scott found that different saponins gave very different results in separating insulin crystals from a crude insulin powder.

It has been observed for many years that blood serum has an inhibitory effect on the hemolysis produced by bile salts and saponin. Ransom (3) attributes this inhibition of blood serum on hemolysis with saponin to the cholesterol in the blood. Bayer

* In this paper, the term "antitoxin" is used for refined diphtheria antitoxin obtained from horse plasma by the Banzhaf method of purification.

(4) found that cholesterol had no effect on the inhibition produced by serum on the hemolytic action of bile salts. He found that lecithin has an inhibitory effect on hemolysis. He also found that euglobulin, pseudoglobulin, and albumin have an inhibitory effect on hemolysis. Ponder (5, 6) pointed out that many of the results which were obtained by the early investigators on the rate of hemolysis were unreliable, since the methods of determining the inhibition of hemolysis were inadequate. Further, he showed that both the blood proteins and the cholesterol in blood serum retard the hemolytic action of saponin and he devised a method of estimating the amount of inhibition quantitatively. Ponder's results point to the inhibition being due to the formation of a loose adsorption compound between the proteins of the blood and the hemolytic agent.

The antitoxin which we used in the following experiments was purified by fractionating diphtheria plasma with ammonium sulfate as in the Banzhaf procedure (7). After dialysis, 60 cc. of the refined antitoxin were diluted to 2 liters with distilled water. This gave a solution containing approximately 0.5 per cent protein. This solution was acidified with acetic acid to pH 6 and allowed to stand overnight. A small amount of protein containing no antitoxin was separated out. The supernatant liquid was decanted off, adjusted to pH 7, and filtered in a Berkefeld filter. The potency of the antitoxin was 150 units per cc. and the nitrogen value was 0.9 mg. per cc. This sample of antitoxin was used in all subsequent experiments. The saponin which was used in these experiments was Merck's Pure White saponin, and was obtained through the British Drug Houses. This saponin was quite active in bringing about the separation of insulin crystals from a crude insulin powder.

pH of Maximum Precipitation of Antitoxin.

It was observed that, when a solution of antitoxin and saponin was acidified, a precipitate developed. In order to determine the acidity at which maximum precipitation occurred, 10 cc. of the antitoxin and 2 cc. of 6 per cent saponin were added to each of a series of 15 cc. centrifuge thimbles. Varying amounts of normal acetic acid were then added to each tube. Immediate precipitation occurred in some of the tubes. The tubes were

then placed in a refrigerator overnight. The following morning, a precipitate had formed in all the tubes and had adhered quite firmly to the glass. The tubes were centrifuged, and pH estimations were made on each of the supernatant solutions with a quinhydrone electrode. The precipitates were allowed to drain for 30 minutes. They were then dissolved in 10 cc. of distilled water, which was made slightly alkaline with N NaOH. The nitrogen was estimated in each of the dissolved precipitates. The protein was calculated by multiplying the nitrogen by 6. The results of a typical experiment are recorded in Table I.

The standard antitoxin before being diluted with saponin and acetic acid contained 5.5 mg. of protein per cc.

TABLE I.

N acetic acid added.	pH	Protein precipitated.
cc.		*mg. per cc.*
0.0	7 0	0.0
0.1	4.5	3 05
0.2	4.15	3.40
0 3	4 0	3.45
0.4	3.9	3.43
0.6	3 8	2.16
0.8	3 7	1.57

It would appear from Table I that maximum precipitation of the antitoxin occurred at about pH 4. In a series of control tubes of antitoxin, to which no saponin had been added, no precipitate formed on standing overnight at pH 4. Some difficulty was experienced in carrying out the nitrogen estimations, because of the large amount of carbon which was present. This would suggest that a part of the saponin was precipitated with the antitoxin.

Estimation of Saponin Adsorbed on Precipitated Antitoxin.

The method of estimating saponin depends on the ability of this substance to hemolyze washed blood cells. The two most common methods of estimating saponin are, first, the one in which the per cent of hemolysis is estimated at the end of 2 hours, as in the method of Vestlin (8), secondly, the method in which the

time dilution curve is plotted for complete hemolysis, as worked out by Ponder. We have tested both these methods for estimating saponin. We prefer the Ponder method, because of the quickness with which an estimation can be made, and also because of the accuracy of the results obtained. We have followed in detail the technique as worked out by Ponder (5). The temperature of the bath in which hemolysis was carried out was 31°. The blood from one rabbit was used as the source of blood cells for all hemolytic determinations. The suspension was made up in phosphate-buffered saline (pH 6.9). This would counteract any effect of small amounts of acid or alkali on the rate of hemolysis.

TABLE II.

Saponin.					Protein precipitated.	Ratio $\frac{\text{saponin}}{\text{protein}}$.	Potency	
Amount added	Theoretical.	Experimental.	In supernatant liquid.	Precipitated.			Precipitate.	Supernatant liquid.
cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*		*cc.*	*cc.*
2	2.5	2.49	2.10	0.39	0.31	1.26	<2	120
3	3.75	3.75	2.60	1.15	0.91	1.26	3	110
4	5.0	4.92	2.75	2.17	1.72	1.26	20	70
5	6.25	6.12	3.00	3.12	2.55	1.22	30	50
6	7.5	7.16	3.79	3.37	2.77	1.22	30	50
8	10.0	9.8	6.45	3.35	2.63	1.26	45	40

The original antitoxin at this dilution contains 4.4 mg. of protein per cc. and 120 units per cc.

To each of six 50 cc. centrifuge tubes there were added 40 cc. of the standard antitoxin, and 2, 3, 4, 5, 6, and 8 cc. of 6 per cent saponin, respectively. Water was added to bring the volume to 48 cc. in each tube, and 2 cc. of the solution removed for saponin estimations. All the tubes were then adjusted to pH 4, by the addition of normal acetic acid, and placed in the refrigerator overnight. The following morning, there was a precipitate in all the tubes. The tubes were centrifuged, and the precipitates allowed to drain for 30 minutes. The amount of saponin was estimated in each of the supernatant liquids, and the amount precipitated calculated by difference. The precipitates were dissolved in 50 cc. of distilled water, containing just sufficient N

NaOH to effect solution, and the amount of nitrogen and the potency of the antitoxin were estimated. The nitrogen was determined by the macro-Kjeldahl method. The values recorded are probably slightly higher than the true values, due to the adsorbed liquid. The error, however, is small, because of the nature of this precipitate. The potency of the antitoxin was estimated by intracutaneous testing. All the results are calculated per cc. of mixture. The results from a typical experiment are shown in Table II.

The results of the experiment shown in Table II are most interesting. In the first place they show that saponin is precipitated together with the antitoxin, and, secondly, that the amount of saponin precipitated within certain limits of concentration is practically proportional to the amount of protein precipitated. In other words, within certain limits of concentration, and under certain conditions, saponin and antitoxin are precipitated as if they formed a chemical compound. The ratio of saponin to protein in the precipitates is remarkable constant, in consideration of the methods of assay used. The values obtained for the potency of the antitoxin indicate that there is a considerable loss in potency in all the tubes except the first. The ratio of protein to units of antitoxin remains practically a constant in each of the supernatant liquids and the same as in the original antitoxin. The loss in potency occurs in the precipitate. This means either that the proteins are denatured, or that saponin interferes with the testing of the antitoxin.

Effect of Saponin on Potency of Antitoxin.

To test the possibility of denaturation of the proteins on standing overnight in the refrigerator at pH 4, an experiment was carried out which was similar to the previous one, except that the tubes were allowed to stand for ½ hour at room temperature before centrifuging. In this experiment, the amount of precipitated complex was found to be about four-fifths of the amount precipitated in the previous experiment. The ratio of saponin to protein in the precipitate remained practically constant, and of the same order, namely 1.2. In this experiment, the loss of potency in the precipitate was practically the same as the loss which had occurred when the tubes were allowed to stand overnight.

In another experiment, 40 cc. of antitoxin, which had been acidified to pH 4 and kept in the refrigerator overnight, showed practically no loss in potency. A similar tube, to which 8 cc. of 6 per cent saponin had been added, showed neither a loss in potency nor a precipitate on standing overnight in the refrigerator at pH 7. The saponin content remained the same as in the original solution. A third tube containing saponin and antitoxin was adjusted to pH 4, and left in the refrigerator overnight. The tube was then adjusted to pH 7.5, and stirred until the precipitate dissolved. A loss of about 10 per cent in potency occurred.

There was reason to suppose that the saponin was interfering with the accuracy of the testing, since saponin injected intracutaneously in sufficient amounts produced a skin reaction not unlike that due to diphtheria toxin. Various amounts of saponin were injected into the depilated skin of a rabbit, and it was found that the concentration necessary to produce any reaction was 4 times the greatest saponin content injected in the potency tests in the previous experiments. When 4 times the greatest concentration of saponin found in the previous experiments was added in diluting antitoxin for a potency test, no effect on the potency of the antitoxin was observed. No potency tests were carried out by the Ehrlich method, as it was thought that the same objections might be made to the injection of saponin.

Effect of Adding Sodium Chloride to the Antitoxin.

In a preceding experiment, it was noted that a certain quantity of saponin had to be added to the antitoxin before any precipitation occurred at pH 4, and, also, that the antitoxin was not completely precipitated even when there was a considerable excess of saponin present. It occurred to us that the presence of a small amount of electrolyte might partially explain this phenomenon. Accordingly, an experiment was carried out in which known amounts of sodium chloride were added to the antitoxin. To a series of tubes containing 40 cc. of the standard antitoxin were added the following amounts of sodium chloride, 0.25, 0.50, and 0.75 gm. respectively. 6 cc. of 6 per cent saponin were added to each tube. The acidity was then adjusted to pH 4, and the tubes placed in a refrigerator overnight. The following morning it was observed that no precipitation had occurred in any of the tubes.

Saponin estimations were made on each tube. The results of one experiment are recorded in Table III.

In this experiment, it was observed that the presence of 1, 2, or 3 per cent sodium chloride completely inhibited the precipitation of the saponin-antitoxin complex at pH 4. The results of the saponin estimations indicate that sodium chloride prevented the formation of such a compound. The inorganic material in one tube containing 40 cc. of antitoxin and 6 cc. of 6 per cent saponin was found to be 0.03 per cent. This value is probably somewhat low, owing to the fact that ammonium sulfate would volatilize during the ashing. Nevertheless, this amount of salt would have some inhibitory effect on the formation of the saponin-antitoxin complex.

TABLE III.

Antitoxin volume.	NaCl	Acidity.	Saponin content.
cc.	*per cent*	*pH*	*per cent*
40	0	7	0 80
40	1	4	0 77
40	2	4	0 77
40	3	4	0 77

Action of Saponin on Insulin.

In view of the fact that saponin has recently been used to effect the separation of insulin crystals, it seemed desirable at this stage to carry out a few experiments on insulin. The insulin used in these experiments contained 15,000 units per gm. To 40 cc. of insulin solution, pH 7, containing 0.4 per cent protein, were added 8 cc. of 6 per cent saponin. This ratio of saponin to protein was found to yield insulin crystals under the conditions worked out by Harington and Scott. Two similar tubes of insulin and saponin were adjusted to pH 4 and 5.6 respectively. The tubes were then placed in a refrigerator overnight. The following morning they were centrifuged and the saponin content of the supernatant liquid and the protein in the precipitates were estimated. The average values from two estimations are given in Table IV.

The results of these experiments indicate that the action of

saponin on the insulin protein depends on the acidity at which the experiment is carried out. At pH 5.6, the isoelectric point of insulin, there was no detectable precipitation of the saponin with the protein. The amount of protein precipitated in these experiments is also much less than in the experiments with antitoxin. Saponin, in these experiments, would seem to have the property of a solvent rather than of a precipitating agent as in the experiments with antitoxin.

TABLE IV.

pH	Saponin.			Protein precipitated.	Ratio $\frac{\text{saponin}}{\text{protein}}$.
	In mixture.	In supernatant liquid.	Precipitated.		
	mg. per cc.	*mg. per cc.*	*mg. per cc.*	*mg. per cc.*	
5.6	7 8	7.8	0	1 89	
3.95	7.8	6.1	1.7	1 4	1.2

TABLE V.

	Saponin.	Protein.	Ratio $\frac{\text{saponin}}{\text{protein}}$	Potency.
	mg. per cc.	*mg. per cc.*		*units per cc.*
Original mixture....	7.8	4.6		127
1st supernatant fluid....	3.8			
1st precipitate....	4.0	3.1	1.3	37
2nd supernatant fluid....	2.4			
2nd precipitate....	1.6	1.3	1.2	26

Nature of the Saponin-Antitoxin Complex.

Ponder has suggested that saponin forms a loose adsorption compound with blood protein. If such is the case, it might be expected that by dissolving the precipitated saponin-antitoxin complex in a solution which is alkaline to litmus, the saponin would be set free. An experiment was carried out to test this possibility. To 40 cc. of antitoxin were added 6 cc. of 6 per cent saponin, and the acidity was adjusted with normal acetic acid to pH 4. The flask was then placed in a refrigerator overnight. The following morning the precipitate was removed by means of a centrifuge. The supernatant liquid was decanted off, and

the saponin content estimated. The precipitate was allowed to drain for ½ hour and was then dissolved in 40 cc. of water, containing 0.8 cc. of N NaOH. This solution was alkaline to litmus. It was then acidified with 1.2 cc. of N acetic acid and immediately a precipitate settled out. The tube was allowed to stand overnight, and the precipitate was removed by means of the centrifuge. The amount of saponin was estimated in the supernatant liquid and the protein determined in the precipitate. The results of this experiment are given in Table V.

In this experiment, the amount of saponin in the precipitates was estimated by difference. The results show that a great part of the saponin can be liberated by dissolving the precipitate in an alkaline solution. It was also interesting to note that a second precipitate formed at pH 4, without the further addition of saponin. This second precipitate, although much smaller, had a ratio of saponin to protein which was in the same order as the ratio in the first precipitate. In another experiment the first precipitate was dissolved in 40 cc. of 2 per cent sodium chloride at pH 7. The saponin content was estimated. The value obtained was equal to the amount which had disappeared from the liquid. These experiments would indicate that the complex of saponin and antitoxin which forms at pH 4 is a loose combination.

DISCUSSION.

It has been shown that when an active saponin is added to a dilute solution of antitoxin, and the solution slightly acidified with acetic acid, a precipitate forms. Maximum precipitation occurs at pH 4. The amount of precipitate depends upon the amount of saponin added, up to a concentration of 0.75 per cent. Further, the ratio of protein to saponin in the precipitate was found to be practically a constant, and within limits, to be independent of the amount of precipitate formed. The ratio of potency to protein in the supernatant liquid remained the same as in the original antitoxin. This would indicate that no purification was effected by the fractional precipitation of antitoxin with saponin. The loss in potency occurred in the precipitate. An experiment was carried out in which the tubes were allowed to stand ½ hour at room temperature. About 30 per cent of the potency could

not be accounted for in this experiment. In a control experiment, to which there had been added no saponin, there was no loss in the potency of the antitoxin at pH 4, when kept in the refrigerator overnight. In an experiment carried out at pH 7, there was no complex formed, nor was there a loss in potency. The results would indicate that the union between saponin and antitoxin is a very loose combination. The saponin can be liberated by merely dissolving the precipitate in a solution which is alkaline to litmus, or in a 2 per cent sodium chloride solution at pH 7. In all these experiments, it was noted that a certain amount of saponin had to be added to the antitoxin before any precipitation occurred at pH 4. This phenomenon could, in part, be explained by the presence of electrolytes. It was found that 1 per cent sodium chloride completely prevented the formation of a saponin-antitoxin complex. An experiment with insulin protein, carried out under similar conditions to those with saponin and antitoxin, showed that there was but a small percentage of the saponin and protein precipitated at pH 4. However, the same general ratio of protein to saponin held, namely 1.2. At pH 5.6, no detectable amount of saponin was precipitated with the insulin. A greater proportion of the insulin proteins would have been precipitated at these acidities in the absence of saponin. Thus saponin acted as a solvent for these proteins.

CONCLUSIONS.

1. When saponin is added to refined antitoxin at pH 4 a precipitate occurs. The amount of precipitate depends, within certain limits, upon the amount of saponin added.
2. The precipitate was found to contain both protein and saponin.
3. The presence of sodium chloride prevented the formation of a saponin-antitoxin complex.
4. Saponin can be liberated from the complex by alkali or by a 2 per cent sodium chloride solution.
5. When saponin is added to insulin a saponin-protein complex is formed at pH 4. This complex does not form at pH 5.6.

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STUDIES ON THE EFFFCTS OF OVERDOSAGE OF VITAMIN D.*

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The discovery of irradiated ergosterol as the specific antirachitic substance makes available a concentrated form of vitamin which can be administered to animals in doses of 100 to 500,000 times the daily curative dose. This provides a means for studying the effects of massive overdosages on the body functions and tissues.

Kreitmair and Moll (6), Pfannensteil (7), Harris and Moore (3), and more recently, Klein (5) have found that massive overdosages of irradiated ergosterol produce anorexia, impairment of growth, and a decline in the general physical condition of white rats. Following the administration of extremely high dosages death frequently ensued. Dixon and Hoyle (2) report that the general pathological features mentioned above did not occur when similar dosages were fed to rats on normal diets, but they did find calcium phosphate concretions in the urinary tract.

In view of the results mentioned, it seemed of interest to study the calcium, phosphorus, and ash balances of animals fed varying dosages of irradiated ergosterol for a short time. The present investigation includes the calcium, phosphorus, and ash balances, blood serum calcium and phosphorus, the ash and Ca:P ratio of bones, hearts, and kidneys, and the weight curves of groups fed varying amounts of vitamin D.

Methods.

Four litters of six rats each were divided into six groups. Each group contained a rat from the respective litters, making four

* Read before the meeting of the American Chemical Society at Columbus, May, 1929.

rats in a group. The animals weighed between 40 and 60 gm. These animals were taken at the time of weaning and placed on the following diets with the indicated dosage of vitamin D per day for the last 14 days of the experiment. The duration of the experiment was 4 weeks. Balances were made on the 1st, 3rd, and 4th week of the period.

Group No.		Times daily curative dose.
I	Stock ration* + 0.4 gm. L.F.X.†	40
II	" " " " " + 0.001 mg. ergosterol	50
III	Stock ration + 0.4 gm. L.F.X. + 0.1 mg. ergosterol	1,040
IV	Stock ration + 0.4 gm. L.F.X. + 10.0 mg. ergosterol	100,040
V	Steenbock Ration 2965‡ + 0.4 gm. L.F.X. + 0.001 mg. ergosterol	50
VI	Steenbock Ration 2965 + 0.4 gm. L.F.X. + 10.0 mg. ergosterol	100,040

* Bills' modification of Steenbock formula:

Ground yellow maize	76
Linseed oil meal	16
Crude casein	5
Ground alfalfa	2
Calcium carbonate	0.5
Iodized sodium chloride	0 5

To 3 parts of the above add 1 part of powdered whole milk.

† An irradiated yeast preparation which gave a ++ line test in doses of 10 mg.

‡ Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 274 (1925).

The balances were obtained by determining the difference between the total ash of the food offered and the ash of the urine, feces, and the food unconsumed. The serum calcium was determined by the Kramer-Tisdall (8) method, and the serum phosphorus by the Briggs (1) modification of the Bell-Doisy method. The bone ash determinations were made on extracted fat-free tibia and femur.

Results.

Daily doses of vitamin D up to 1000 times the curative dosage for a period of 2 weeks have no deleterious effect upon the growth of young white rats fed a normal diet. Feeding dosages of 100,000

times the curative dosage has a pronounced effect on the animals. Growth stops in less than a week and loss in body weight ensues. The animals in Group IV lost an average of 11 gm. during the 2 week period in which they received the vitamin D. Groups I, II, and III during the same period gained an average of from 40 to 46 gm. Excessive overdosage has a pronounced effect on the amount of food consumed. During the last week of this experiment the animals in Group IV consumed an average of only 8.25 gm., while those in Groups I, II, and III consumed an average of 65 gm. In appearance, the animals in Group IV were small, emaciated, and had greasy skin and hair. The region around the genitals and anus was stained yellow, a condition previously reported by Harris and Moore. The animals which died from this excessive dosage of vitamin D usually exhibited symptoms of labored breathing, extreme weakness, and recurrent convulsive tremors for 24 hours before death occurred. The animals in Group III, receiving daily 1000 times the curative dosage, appeared normal in all respects.

The results of other work now being carried on indicate that 10,000 times this amount of vitamin D administered daily over a period of 6 months does not affect the growth or general physical condition of the animals. These rats were fed the high calcium-low phosphorus Steenbock Ration 2965.

A similar dosage of vitamin D when administered to nursing mothers fed a modified Steenbock stock ration[1] does not affect the health of the mother or the young, except in those cases in which conditions resembling pellagra appeared. We were able to control this condition by the addition of large dosages of yeast. It has been found that the acute symptoms of vitamin D overdosage can be counteracted at certain levels by liberal feedings of yeast. Experiments now in progress indicate that a continuation of this dosage of vitamin D to second generation animals has no ill effects on their health and reproduction.

Dosages of 50,000 times the normal daily dose of vitamin D do have an effect on the health and reproduction of the mother and young. First generation mothers usually raised several litters, whereas in some cases total failure to reproduce resulted. But the second generation animals on this level of vitamin D failed to

[1] Bills' modification of the Steenbock formula.

reproduce. Some second generation animals maintained on this level of vitamin D showed, at the age of 3 to 4 months, excessive growth of incisors and calcification of some of the organs and tissues, particularly the kidneys, lungs, and aorta.

The effect on the animals fed the rachitogenic diet is not so pronounced. Group VI gained an average of 1 gm., while Group V gained an average of 34 gm. during the 2 week period in which the vitamin D preparation was administered. The animals in Group VI, receiving 100,040 times the curative dosage, showed the same symptoms as those in Group IV. The average food intake during the 4th week was 24 gm. for Group VI as compared

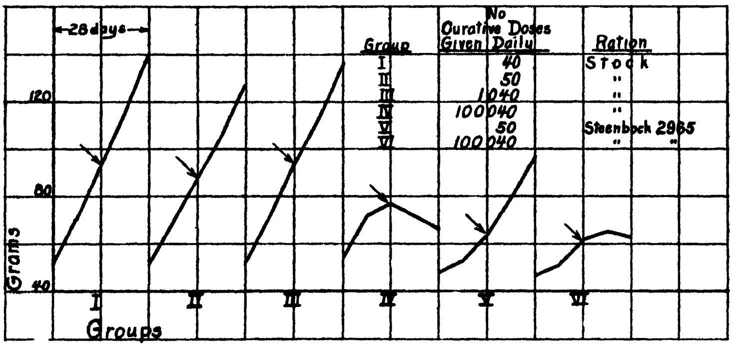

CHART I. The effect of varying dosages of vitamin D on the growth of white rats. Dosages were started at the points indicated by the arrows.

with 85 gm. for Group V which received only 50 times the curative dosage (daily).

The growth curves for the rats in Groups I to VI are shown in Chart I.

The blood serum calcium shows a definite increase over the normal in Groups IV and VI. Blood serum phosphorus shows an increase with increasing dosage of vitamin D in Groups I, II, and III. In Group IV, contrary to expectations, the blood serum phosphorus is lowered. Hess and Lewis (4) have obtained in a few cases an increase in blood serum calcium and a decrease in phosphorus when they administered large dosages of irradiated ergosterol to rachitic children. In Group VI the blood serum calcium and phosphorus are both higher than in Group V, which

substantiates the findings of Klein and other investigators using the high calcium-low phosphorus diet.

The bone analyses (Table I) show a progressive decrease in the percentage of ash and a corresponding increase in the Ca:P ratio with increasing dosage of vitamin D. The percentage of calcium in the ash increases very slightly, while the percentage of phosphorus shows a slight decrease. Consequently the loss of phosphorus is relatively greater than that of calcium, as indicated by the increased Ca:P ratio in the bones of the animals in Group IV. These results indicate a loss of ash from the bone when excessive amounts of vitamin D are administered. Some experiments performed recently corroborate in general the results reported in this paper. The effect of a limited food intake on the depletion of the ash is also being investigated.

TABLE I.

Blood and Bone Analyses.

Group No.	No. of curative doses given daily.	Blood serum.		Bones	
		Calcium.	Phosphorus.	Ash based on extracted dry weight.	Ratio, Ca:P.
		mg. per 100 cc.	*mg. per 100 cc.*	*per cent*	
I	40	11.9	9.2	58 4	1.92
II	50	12 3	9.9	57.0	1 93
III	1,040	12.1	10.3	55.7	1 97
IV	100,040	14.6	8.1	47 5	2 06
V	50	11.7	7.1	51.2	2 00
VI	100,040	15.0	8.0	46.3	2 03

The ash determinations on the kidneys and hearts of the animals show a decreasing percentage of ash with increasing vitamin D intake. One exception to this result was in the kidneys of one rat in Group IV, which contained 6.6 per cent ash consisting of 38.2 per cent calcium and 12.1 per cent phosphorus, and having a Ca:P ratio of 3.2. This high ash content was undoubtedly due to calcium phosphate and other concretions containing calcium. Table II gives the average analyses.

In the calcium and phosphorus balance, calcium retention tends to increase through the 3 weeks of the balance experiment in all

groups except Groups IV and VI, which received the very large dosages, and in these two groups a marked decrease is shown. The phosphorus retention, however, does not change much except in Groups IV and VI, where a marked decrease occurs. In Group IV during the 3rd week there was an actual loss of phosphorus, and during the 4th week a loss of both calcium and phosphorus, indicating a drainage of these elements from the body when massive dosages are administered. In Group VI the Ca:P ratio of the retained calcium and phosphorus increases from 4.0 during the control period to 6.2 and 7.3 respectively for the 2 weeks of the

TABLE II.
Heart and Kidney Analyses.

Group No.	No. of curative doses given daily.	Heart. Ash based on dry weight.	Kidneys.			
			Ash based on dry weight.	Ash.		Ratio, Ca:P.
				Ca	P	
		per cent	per cent	per cent	per cent	
I	40	2.3	3.0	13.1	2.1	6.2
II	50	2.4	3.2	11.0	1.8	6.1
III	1,040	1.7	2.5			
IV	100,040	1.8	3.7*	38.2†	12 1	3.2
V	50	1.8	2.3	12.8	2.1	6.1
VI	100,040	1.0	2.2	21.5	3.6	6.0

* Average of three rats, individuals being 2.7, 6.6, and 1.7 per cent.
† Per cent of Ca and P given is for kidneys having 6.6 per cent ash.

high vitamin D administration. The ratios for Group V are 3.5 for the control week and 4.7 and 4.6 for the last 2 weeks. The relative retention of calcium is about 30 per cent higher than the phosphorus when excessive amounts of vitamin D are administered. It is interesting to note that the positive balance of calcium in Group IV during the third week is greater than the total ash balance which is 24 mg. per animal. These results are given in Table III.

The ash balance shows an increased ash retention throughout the experiment, except in Groups IV and VI in which a decrease is shown, and an actual loss in Group IV for the 4th week. These results are given in Table IV.

Dixon and Hoyle (2), since they found calcium phosphate concretions in the urinary tract, postulate that large overdosages of vitamin D cause an increased absorption of calcium and phosphorus from the intestinal tract and their subsequent elimination through the kidneys. The present study shows that with increasing dosages of vitamin D the percentage of ash in the bones, heart, and kidneys, and the total ash of the entire body decrease.

TABLE III.

Calcium and Phosphorus Balance (Mg. per Rat).

Group No.	1st wk.		3rd wk.		4th wk.	
	Ca	P	Ca	P	Ca	P
I	197.1	148.2	252.9	138.9	277.9	158.8
II	188.6	135.7	218.4	115.5	1152.2	64.7
III	204.0	151.4	237.8	123.3	259.9	147.4
IV	194.1	143.7	67.3	−1.3	−16.8	−31.4
V	293.4	84.7	389.9	82.7	472.4	101.4
VI	358.8	88.7	231.3	37.3	266.3	36.4

TABLE IV.

Ash Balance (Mg. per Rat).

Group No.	1st wk.	3rd wk.	4th wk.
I	860.4	943.7	1183.0
II	899.0	804.0	455.0
III	970.0	843.0	1109.0
IV	1029.0	24.0	−214 0
V	1034.0	1266.0	1723 0
VI	1224.0	711.0	859.0

The blood serum calcium and phosphorus and the Ca : P ratio of the bone ash show a progressive increase, with the exception of blood phosphorus in Group IV. The calcium and phosphorus and ash retention decrease with the high dosage. These results indicate that there is a drainage of mineral constituents from the body when excessively high dosages of vitamin D are administered and that there is a relatively greater elimination of phosphorus than of calcium from the body. If phosphorus, as suggested by Dixon and Hoyle (2), is eliminated through the urinary tract, it must be

in a soluble form, possibly as secondary calcium phosphate or combined with sodium or potassium. Should this be true, then the excessive elimination of phosphorus through the kidneys might account for the loss of minerals other than calcium and phosphorus.

These experiments indicate that excessively large dosages of irradiated ergosterol must be administered before any ill effects are noted. Other experiments show that amounts as high as 10,000 times the daily curative dose given over a long period of time have no effect on the growth of white rats and no apparent effect on their body functions.

The wide range between the effective amount of vitamin D required for the health of the animal and the daily intake which has deleterious effects on the growth of white rats is indicated by these results. If it were possible to apply these relative figures of the toxic dose of vitamin D for white rats to the infant, the harmful dose would be 1.5 liters per day of a solution of irradiated ergosterol in oil 100 times as potent as cod liver oil.

CONCLUSIONS.

1. Excessively large dosages of irradiated ergosterol must be administered daily before any ill effects are noted.
2. Dosages as high as 10,000 times the daily curative dose over a period of 6 months have no effect on the growth of white rats, and no apparent effect on their body functions.
3. Excessive amounts of vitamin D cause a drainage of mineral constituents from the body, with a relatively greater elimination of phosphorus than of calcium.
4. Dosages of 100,000 times the daily curative dose produce anorexia, emaciation, greasy hair, labored breathing, and eventually death.

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THE TESTICULAR HORMONE.*

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Preparation.

The preparation of testicular extracts has for the last 3 years been subjected to intensive investigation by this laboratory. McGee (1) and McGee, Juhn, and Domm (2), have shown that extracts of the lipid fraction of bull testicles exert a striking effect on the secondary sex characters of the Brown Leghorn capon. Gallagher (3) has shown that by the present methods of extraction and assay this activity is found in no other tissue save testis and epididymis. Moore and McGee (4) have shown these extracts to possess the same activity as the internal secretion of the testis by their effect on the spermatozoa in isolated epididymides according to the criterion proposed by Moore (5). These results in the mammal have been extended by Moore and Gallagher (6) with more highly purified preparations and some attempt has been made to investigate this reaction as a means of quantitative assay. A further new series of indicators has been proposed by Moore and Gallagher (6, 7), Moore, Price, and Gallagher (8), and Moore, Hughes, and Gallagher (9), based upon cytological changes in the accessory reproductive organs and the secretion of the seminal vesicles and prostate gland.

These varied biological manifestations of the testis hormone have been developed by the Chicago group in hope of attaining some rapid quantitative method of assay for testicular extracts. An extensive investigation of the comb growth reaction in the

* These studies were in part supported by a grant from the Committee for Research in Problems of Sex of the National Research Council. We wish to express our appreciation to Professor Frank R. Lillie for making this possible.

Brown Leghorn capon has been undertaken by Gallagher and Koch, which will be the subject for a future communication. It will suffice here to note that while this reaction admits of certain quantitative interpretation it is by no means as accurate as desired. It fulfils however the requirement of rapidity in that but five daily injections are required.

In the studies reported on in this paper the routine assay has been the injection of the extract once daily for 5 days. We wish to thank Dr. L. V. Domm of the Whitman Laboratory of Experimental Zoology for preparing the capons used in these investigations. Comb measurements are taken on the 1st, 3rd, and 6th days and results interpreted on the basis of the preliminary standardization of the extract studied. It must be emphasized that proper interpretations can be made only if a minimal dose be determined and all subsequent studies be based on this minimal dose.

The method of preparation proposed by McGee has been investigated and adopted by us as the first step in the routine extraction.

The tissue is ground and extracted with 4 volumes by weight of 95 per cent alcohol for from 3 to 5 days. The alcoholic extract is pressed out, concentrated to a sludge under diminished pressure, and extracted with benzene.

In view of the paucity in yield of activity per unit weight of tissue we investigated at some length the completeness of our alcoholic extraction. Since reextraction of the tissue residue with 95 per cent alcohol yields very little or no activity, we have found no justification for reextracting the tissue as a routine precaution. Womack in this laboratory is extending these results.

Another explanation of the low yield suggested itself; namely, that the active principle might be bound in the tissue in such a way that the activity might not be recovered completely by a simple extraction process. Hydrolysis with strong alkali, acids and enzymes was investigated but in no case could the yield be increased. There was often some loss, especially with alkali treatment, but it is significant that fresh bull testis may be boiled with 40 per cent NaOH for 2 hours without too great loss of activity. This finding however does not disprove that the active principle is in some way bound, for, since there was some destruc-

tion, there may have occurred simultaneous release and then destruction of the activity.

The benzene-soluble material as obtained by the procedure adopted by McGee (1) is unfit for continued injection due to the severe local reaction produced. The most satisfactory treatment consists in complete removal of benzene by distillation under diminished pressure and treatment of the residue with acetone. The complete removal of benzene is of great importance. We have repeatedly noted that if the removal is not complete the yield of solids obtained in the acetone varies considerably and the complete removal of activity from the lipid precipitate is not obtained. The acetone-soluble material contains the greater part of the

TABLE I.

Assay of Preparation 84.

Preparation.	Weight of yield.	Dosage.	Assay.
	gm.		
Alcohol-soluble.		Very large, toxic.	None made.
Benzene-soluble.	211	" " "	" "
Acetone-soluble.	20	0 010 gm.	Negative, 3 birds.
		0.015 "	" 3 "
		0 020 "	" 3 "
		0 030 "	Positive, 3 "
Acetone-insoluble.	191	0 15 "	Negative.

activity. Some slight activity may be recovered by a second extraction of the acetone precipitate, but when this is done, the activity per unit weight is distinctly decreased. The material obtained in this step is suitable for repeated assay in the bird but is toxic in small doses to the mammal. The yield is usually of the order of one-tenth the total weight of the benzene-soluble substance and the minimal daily dose in the capon of the product varies from 20 to 30 mg. The details followed in an actual experiment are given below.

Preparation 84.--29 kilos of tissue were extracted with alcohol, concentrated, and extracted with benzene as outlined. The benzene-soluble material weighed 211 gm. This was extracted with 1 liter of acetone at $-10°$ for 24 hours. The acetone-

soluble material weighed 20 gm. Table I gives the results of the assay. The minimal daily dose hence is the equivalent of 43 gm. of frozen bull testicle.

This material is rich in cholesterol and neutral fat. Several alternative procedures have been investigated for further purification. Precipitation with dilute alcohol suggested itself. By using 50 per cent alcohol at $-10°$ roughly 96 per cent of the solids may be precipitated. The filtrate is highly active, the minimal dose varying from 0.2 to 2 mg., dependent upon conditions which we shall discuss. The recovery in activity however is not entirely satisfactory, varying from 40 to 60 per cent. Even after five extractions of the precipitate much activity still remains. The principal difficulty is met in the physical state of the precipitate. Emulsions form which presumably adsorb the active substance and a layer of liquid fat usually separates which tends to hold back the hormone.

70 per cent alcohol at room temperature offers greater advantage. The yield in solids is about 40 per cent, but the recovery of activity is fairly complete. The material may be further purified by using smaller volumes of alcohol and repeating the procedure with the soluble material. Here, however, the yield in activity begins to fall, complete recovery in the desired fraction being impossible. Since these procedures have been discarded, for the sake of brevity, no typical experiments will be given here.

Various experiments involving partition between immiscible solvents were undertaken, the most satisfactory one being a separation of the acetone-soluble material between 3 volumes of 70 per cent alcohol and 4 volumes of hexane. The yield in solids is approximately 4 per cent and the recovery of activity from 70 to 80 per cent. We have accepted this loss in view of the relatively great purification attained.

The material now is dissolved in ether as well as possible and washed with 10 per cent NaOH. The yield in solids in the ether phase is from 15 to 20 per cent and as far as we can determine complete activity is recovered. Details of a typical experiment are given.

Preparation 89.—36 gm. of acetone-soluble material obtained by the routine procedure were dissolved in 175 cc. of hexane and then shaken with 125 cc. of 70 per cent alcohol. The 70 per cent

alcohol layer was washed five times with hexane. The total hexane-soluble material was then reextracted twice with 75 cc. of 70 per cent alcohol and in each case the alcohol layer was washed five times with fresh hexane. The 70 per cent alcohol-soluble material after removal of the alcohol was transferred to ether and shaken with 10 cc. of 10 per cent NaOH. The NaOH solution was washed five times with fresh portions of ether and the ether solution shaken repeatedly with water. The ether-soluble material is then suitable for assay. The result of the assay is shown in Table II.

The material thus obtained may by suitable treatment be rendered water-soluble, but our data are as yet incomplete and we prefer to postpone communication on this phase of the problem.

TABLE II.

Assay of Preparation 89.

Preparation.	Total weight.	Daily dose.	Assay.	Recovery of activity.
	gm.			*per cent*
Acetone-soluble.	36	0.03 gm.	Active.	
		0.02 "	Inactive.	
Hexane-soluble.	34.8	0.10 "	"	
		0.15 "	Trace of activity.	15 (?)
70 per cent alcohol-soluble.	1.28		None made.	
Ether-soluble.	0.285	0.28 mg.	Active.	85 (?)
		0.50 "	Strongly active.	

Further purification may be obtained by precipitating the ether-soluble material with small quantities of a low boiling hydrocarbon such as pentane. There is however always a decided split in the activity, and a consequent low yield. This is rather to be expected since our data indicate a rather low solubility of the hormone in such solvents.

DISCUSSION.

The method as outlined has certain features both in its favor and against it. The long treatment and difficulty of handling such extractives quantitatively speak against it. On the other hand, a potent preparation in fairly good yield may be obtained.

The material upon repeated injection in the bird is certainly non-toxic and in the mammal even huge doses elicit no unfavorable local reaction.

The solid content of the daily minimal dose for the capon may be reduced as low as 0.01 to 0.03 mg. if complete recovery of activity is not desired. In such a case the volume of 70 per cent alcohol used may be reduced and quite a difference in the yield of solids effected. In many chemical studies we have preferred to emphasize purity of product rather than completeness of yield. These will be a subject for future communication.

Whether one or more active principles are concerned is at present mere speculation. It is however true that by one and the same method of preparation an extract is obtained which will exert all the biological effects thus far postulated for the testis hormone. Physiological and morphological criteria have been proposed and in every case complete substitution may be obtained with the testis extract here described.

It is our feeling that until more is known of the chemical nature of the hormone no name should be given the extract. As yet any name would be valueless and not at all descriptive. Too often a name gives a false sense of security as regards the purity of the product, a fact we wish to emphasize, for it is our firm opinion that the extract is as yet grossly impure even though the minimal daily bird dose be as low as 0.01 mg.

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RICKETS IN RATS.

X. FASTING TETANY AND PHOSPHATE TETANY.

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When rats made ricketic on a high calcium-low phosphorus regimen are offered a diet containing a normal amount of phosphorus, the blood serum calcium is lowered and the inorganic phosphorus raised, the rickets heals rapidly (1), and tetany develops (2). Wilder (3) has recently confirmed the former reports (4–7) that fasting also heals the rickets, and produces a similar blood picture. He states that tetany is present also. He made additional observations on the distribution of the phosphorus in the blood and shows that the phosphorus comes from the destruction of the tissues and not from the red blood cells. He questions whether the condition described by us is due to the phosphorus in the diet and implies it is due to the reduced food intake. His contribution has raised several questions, the solution of which should aid in the understanding of the pathogenesis of tetany.

The convulsions of the fasting ricketic animals observed by him were not mentioned by McCollum and coworkers (4) nor by Kramer and Howland (5). Cavins (6) states that only one of his animals had convulsions, and Harrison (7) did not observe convulsions in a large series of animals. Wilder does not detail how frequently convulsions occur but in a personal communication states:

" . . . the reason that Miss Harrison did not observe convulsions may have been that her animals had a milder rickets (?). Our animals all had rather severe rickets, and although most of such animals behaved very dependably, a few did not develop tetany, grossly at least.

As to the evidences of tetany, they were very dramatic, often nearly constant twitching (more of the fore legs than the rear), with the toes

curled in. When such animals appeared quiet a puff of breath blown at them would cause them to jump an inch or so in the air and then fall on their sides with all four legs twitching convulsively for a few seconds. Palpation of the muscles about the jaw revealed spasmodic twitching of those muscles. The increased spasticity of all their muscles appeared to interfere markedly with locomotion. In other words the signs were so well marked that we made no attempt to do electrical reactions. We used the term 'convulsions' in the paper no doubt when 'tetany' would have been better, to designate active twitching, rather than a latent type of thing. We did not wish to imply any demonstrated parallel between that type of tetany however and the spasmophilic tetany of infancy."

The question arises, is this condition tetany? A quantitative test of the neuromuscular response to the galvanic current (8) if positive, would make this diagnosis sure.

Secondly, what causes the tetany? The studies of Binger (9) and Salvesen (10) leave little doubt that phosphate may cause tetany in normal animals. It has been our thesis that ricketic animals are especially susceptible to the effect of phosphates. Ricketic children and ricketic rats were thrown into tetany (1) by amounts of phosphate which had no effect on non-ricketic animals (11). Ricketic dogs (unpublished data) developed tetany upon doses of phosphate which have no effect on normal dogs. Wilder, in confirmation of Harrison offers further supporting evidence when it was shown that when normal rats are fasted they do not have a raised inorganic phosphorus of the blood serum nor do they have convulsions. They have the same loss of weight and hence the destruction of muscle tissue should produce the same amount of phosphorus for absorption into the blood stream. The problem is then rather reversed, not whether phosphate tetany is due to fasting but whether fasting tetany is due to phosphate.

Thirdly, does tetany occur because of lack of food, or can animals in tetany not eat? Wilder (3) has stated:

"In 1927, Karelitz and Shohl also observed a similar phenomenon after adding phosphate to the rickets-producing diet of rachitic rats, though it is noteworthy in this connection that their animals refused the phosphate diet to the extent of considerable loss in weight. As we shall show, the type of results reported by these last authors can be produced by fasting rachitic animals. This fact casts doubt on the supposition of Karelitz and Shohl that ingestion by rachitic rats of large amounts of phosphate effects fundamental changes in methods of phosphorus absorption and excretion. That

is to say, no allowance was made in their experiments, otherwise very conclusively worked out, for the large quantity of phosphorus released from body tissues during fasting."

In 1927 (12) we published a further paper which Wilder had unfortunately overlooked. We stated: "The purpose of the present study was to determine the importance of restricted food intakes on the healing of rickets, and to consider further [the effect upon] the metabolism of calcium and phosphorus." Under these conditions no corresponding blood changes occurred and rickets was not healed. This we may call a "negative" control.

We were forced to this type of procedure because at that time we did not know how to make the animals eat more of the food containing excess phosphate. If an experiment could be devised in which the animals gained weight, the "fasting effect" would be completely eliminated. The conclusion would then follow that tetany and the healing of rickets was produced by the phosphate eaten. This paper offers such a "positive" control.

Several types of procedure are available: (*a*) the food may be shifted gradually; (*b*) less phosphate may be added; (*c*) the acid-base content may be altered; (*d*) the fasting and feeding may be compared for the relative time of onset of symptoms, the food intakes and weight and electrical reactions being carefully observed.

Plan of Experiment.

Ten animals from one litter whose mother was fed the Sherman Diet B (1) were weaned at 21 days and continued upon the same diet. At 30 days of age they were fed the Steenbock and Black ricketogenic Ration 2965.[1] 21 days later they were x-rayed and found to have marked rickets. They were then divided into five groups of two each. The first pair was fasted (water only). The second pair was given enough phosphate (as NaH_2PO_4) to change the ratio of the Ca:P from 5.0 to 2.0. The third and fourth groups were given a diet with a ratio of 2.0 on the 1st day and 1.0 on the 2nd day. The former was made with Na_3PO_4 and the second with H_3PO_4. The last group (Group E) was maintained upon the original diet as controls. The experiment was begun at noon after the electrical reactions were

[1] Steenbock, H., and Black, A., *J. Biol. Chem.*, **64**, 274 (1925).

TABLE I.

Effect of Fasting and Added Phosphate upon Ricketic Rats.

Rat No.	Diet.	Time.	Weight.	Food intake.	Electrical reactions,* in milliamperes. C.C.	A.C.	A.O.	C.O.	Average.	Remarks.
		hrs.	*gm.*	*gm.*						
2318	Fasting.	0	66 5	0	0.24	0.24	0.48	0.64	0.40	
		5	65.8	0	0.26	0.24	0.44	0.52	0.36	
		10	64.0	0	0.24	0.18	0.44	0.60	0.36	
		22	60.0	0	0.10	0.12	0.20	0.22	0.16	
		28	60.0	0	0.06	0.20	0.20	0.14	0.15	
		34	59.0	0	0.06	0.12	0.12	0.14	0.11	Tetany.
		46	56.0	0						
		52	56.0	0	0 10	0.14	0.08	0.22	0.13	
2320	Steenbock + NaH_2PO_4; Ca:P = 2:1.	0	56.5	0	0.34	0.36	0.84	0.68	0.55	
		5	58.8	0	0.32	0.22	0.56	0.70	0.45	
		10	56.0	1.5	0.32	0.14	0.32	0.52	0.32	
		22	56.0	3.0	0.16	0.16	0.32	0.28	0.23	
		28	56.0	3.0	0.16	0.18	0.32	0.30	0.24	
		34	56.0	3.5	0.14	0.14	0.18	0.20	0.16	Tetany.
		46	55.0	5.0						
		52	54.0	5.0	0 08	0.28	0.32	0.24	0.23	
2322	Steenbock + Na_3PO_4; Ca:P = 2:1 1st day, 1:1 2nd day. Alkaline.	0	69.0	0	0.64	0.48	0.56	1.00	0.67	
		5	72.0	1.5	0.28	0.26	0.40	0.60	0.39	
		10	73.0	3.5	0.10	0.10	0.20	0.30	0.17	
		22	70.0	4.5	0.24	0.22	0.32	0.50	0.32	
		28	69.0	5.5	0.20	0.20	0.40	0.36	0.29	
		34	68.0	5.5	0.20	0.26	0.41	0.41	0.32	
		46	65.0	5.5						
		52	63.0	6.0	0.02	0.04	0.04	0.02	0.03	Marked tetany.
2324	Steenbock + H_3PO_4; Ca:P = 2:1 1st day, 1:1 2nd day. Neutral.	0	52.0	0	0.38	0.36	0.60	0.64	0.49	
		5	55.3	0.5	0.22	0.20	0.32	0.62	0.34	
		10	55.5	2.5	0.60	0.42	0.96	0.72	0.67	
		22	54.0	3.5	0.34	0.22	0.40	0.70	0.41	
		28	53.5	5.0	0.18	0.20	0.26	0.46	0.27	
		34	52.5	5.5	0.20	0.28	0.36	0.42	0.31	
		46	51.0	5.5						
		52	50.5	5.5	0.06	0.08	0.08	0.10	0.08	Marked tetany and carpopedal spasm.

TABLE I—*Concluded.*

Rat No.	Diet.	Time.	Weight.	Food intake.	Electrical reactions,* in milliamperes. C.C.	A.C.	A.O.	C.O.	Average.	Remarks.
		hrs.	*gm.*	*gm.*						
2326	Steenbock; control.	0	58.5		0 42	0.72	0.72	0.92	0 69	
		5	60.5		0.56	0.44	0.70	1.00	0.68	
		10	62.0		0.48	0.28	0.60	0.72	0.52	
		22	59.0		0.36	0.32	0.96	1.00	0.66	
		28	59.0		0.28	0 40	1.00	0.78	0.61	
		34	58.0		0 32	0.20	0.36	0.56	0.36	
		46	58.5							
		52	58.0		0.44	0.24	0.96	0.80	0 61	

* C.C., cathodal closing; A.C., anodal closing; A.O., anodal opening; C.O., cathodal opening.

determined on all. The animals were weighed, the food consumption measured and the electrical reactions determined 0, 5, 10, 22, 28, 34, 46, 52 hours later. The animals were then killed and bled under light ether anesthesia, and by the method of Kuttner and Cohen (13) the calcium and phosphate of the serum were determined.

In addition to the above we shall mention numerous other experiments in which these determinations were made but not all upon the same animals.

Results.

The results of the electrical reactions are given in Table I.[2] The data show that fasting animals develop a condition which should clearly be called tetany. *No gross convulsions* were seen but only a fine tremor. This condition develops later than the phosphate tetany. Phosphate tetany was evident in less than 8 hours. The tremor which was similar to that of the fasted animals developed early. Later two of the animals developed carpopedal spasm of one or more of the extremities and were unable to use them in walking. When picked up a fine tremor of the whole body could

[2] At the request of the Editors only a single protocol of each type is given. The results for the second animal were quite similar.

be felt. A clap of the hands induced a transient generalized convulsion. During this time the animals had eaten the food and gained weight. The controls, as is usual after 3 weeks upon this diet, show slight loss of weight but no tetany. Therefore the condition is obviously due not to fasting but to the food eaten.

We have seen animals die within 12 hours when shifted to a high phosphate diet after they had eaten only 0.6 gm. of food and gained weight. Fasting, which shows no effect for more than 24 hours, could have played no part.

Wilder has reported convulsions in fasting ricketic rats with losses in weight *usually* greater than 5 gm. per day. Harrison's weight curves show a loss of 5 gm. per day. On numerous occa-

TABLE II.

Effect of Fasting and Added Phosphate upon Blood Serum of Ricketic Rats.

Rat No.	Diet.	Calcium.	Phosphorus.
		mg. per cent	*mg. per cent*
2317-18	Fasting.	7 5	9 2
2319-20	Steenbock + NaH_2PO_4; Ca:P = 2:1.	8 3	9 1
2321-22	Steenbock + Na_3PO_4; Ca:P = 2:1 1st day, 1:1 2nd day. Alkaline.	7 6	9 8
2323-24	Steenbock + H_3PO_4; Ca:P = 2:1 1st day, 1:1 2nd day. Neutral.	7 9	8 8
2325-26	Steenbock; control.	11 3	3 5

Rats were killed after 52 hours on the specified diet.

sions tetany has been observed with a loss in weight of only 1 or 3 gm. when phosphate was fed. Some animals show more tetany than others. If they do not die and recover from the tetany, they eat well.

The blood analyses of the experimental animals are given in Table II. The specimens were obtained after 52 hours on the various diets. Compared with the ricketic controls they show a lowered calcium and increased inorganic phosphorus of the blood serum. Under the modified, less severe conditions the changes are not so marked as under those originally reported, but represent a similar mobilization of calcium and phosphorus.

DISCUSSION.

Our conditions have resembled Harrison's more closely than Wilder's, a description of which we feel should be included as a

possible reason why he observed a more severe condition than those here reported.[3]

The clinical manifestation described by Wilder and the blood analyses make tetany the probable diagnosis but the electrical reactions prove it *correct*.

It is our impression that ricketic animals are self-fasted. If they continue to eat the food, they continue in tetany. By fasting

[3] We quote from a personal communication of Dr. Wilder.

"As to the age of the rats used in our experiments. Probably our animals in general were on the diet somewhat longer than yours. To secure marked rickets, as evidenced by x-ray, I found it necessary to keep 35 gm. rats on Diet 3143 (McCollum) for at least 6 weeks. At the end of that time the blood inorganic phosphate was usually below 3 mg. (whole blood). If such rats were kept on the diet as long as 8 weeks however they entered an inanition phase marked by decreased activity, stationary weight, loss of appetite, and incidentally an elevation of the blood phosphate to 7 or 8 mg., which I ascribe to their decreased food intake,—a partial fast. The rats used for Table I [*cf.* (3)] were killed probably between their 10th and 12th weeks of life, having been on the rachitic diet 6 to 7 weeks. Table II [*cf.* (3)] gives only a few examples from a large number of experiments. Many of the latter had no nitrogen measurements, but in other respects were identical with the ones cited. You will note that one of the rats of Table II weighed 173 gm. at the time of the experiment. This rat and two others were made rachitic, then given a small amount (one dose) of cod liver oil but kept on their rachitic diet with the result that when they were between 4 and 5 months old they were again severely rachitic, as shown by x-ray in themselves, and by blood inorganic P in other rats similarly treated. Because of their age it took these rats longer, than it does young rats, to develop rickets for the second time. Naturally, because of their size these large rats were more suitable then small ones for unit metabolism experiments. Another way we obtained large rachitic rats was to put normal rats of 50 gm. on the rachitic diet and allow them somewhat longer than usual to develop their rickets, checking up in all cases with x-rays of the bones. The large rats developed just as severe convulsions as did the young ones.

The survival period of the rats was almost invariably only 3 or 4 days and the three reported that lived longer represent the only ones, out of at least thirty I should say. Obviously, rats that died in 48 hours or so gave us little information from urine N and P, and we would gladly have prolonged the period of tetany for purposes of study, if we had been able to. It may be, that since the three that survived longest were exceptions, we should not have given them the prominence we did; however, fundamentally, or at least, so far as we could tell chemically, they went through the same reactions as did the ones that died more rapidly."

they may produce phosphorus but they also produce acid and this tends to heal the tetany or prevent it (14, 15). In any event they are between the two horns of the dilemma. If they eat they get phosphorus; if they fast they get phosphorus. The phosphorus from 5 gm. of muscle is approximately 10 mg. per day. They get the same amount from 1 gm. of food of Ca : P of 1.0.

Animals that lose weight do not necessarily have tetany. When we fed high phosphate with an excess of acid (16) the rats lost more weight than the other groups but did not develop low electrical reactions. Acidosis tends toward dehydration (14) and how much is due to loss of body water and body tissue is a complicated problem. The assumption is that such animals lose as much tissue but the acidosis cures or masks the tetany (15).

If they eat enough to kill themselves at one meal, fasting cannot be said to play a part.

From this study it seems probable that the tetany of fasting is due to phosphorus and is a related phenomenon to that described as phosphate tetany.

CONCLUSIONS.

1. Fasting of ricketic rats as measured by the response to the galvanic current results in tetany. It is probably due to phosphate.

2. Tetany due to feeding food which contains added phosphate can be demonstrated in animals gaining weight. It develops soon after the food is eaten. The condition is properly called phosphate tetany.

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Note by T. S. Wilder.

In complying with the courteous suggestion of Dr. Shohl that I add a note to his paper I shall attempt to be as brief as possible.

Our paper attempted to show that, in fasting rachitic rats, the inorganic phosphate which enters the circulation from endogenous sources, probably muscle, may be regarded as the primary cause of the tetanic condition found in such animals. Hand in hand with the high blood phosphate we observed an excessive excretion of phosphate in the urine. This finding led us to comment on that part of the paper of Karelitz and Shohl (*J. Biol. Chem.*, **73**, 665 (1927)) which dealt with the metabolism of phosphate in rachitic rats to whose diet phosphate was added. It appeared to us that since those rats refused food to the extent of a marked loss in weight there must necessarily have been a certain amount of phosphorus released into the circulation from body tissues. In other words, we felt that there were two possible sources for the high urinary phosphate responsible for the reversed excretion ratio between stools and urine. The magnitude of the endogenous supply is indicated by the fact that one of our rats excreted over 13 mg. of phosphate in the urine in 24 hours (and this after its serum phosphate was raised from about 3 to about 13 mg. per 100 cc. and perhaps a small amount deposited in the bones). It would appear from this that there was a potential endogenous supply nearly equaling the amount of phosphorus contained in 1.9 gm. of food of 1 per cent phosphorus content, and probably exceeding the amount that would be absorbed from the food. No doubt this maximum figure is much larger than the amounts of phosphorus released from tissues in semifasting. Semifasted rachitic rats do not usually develop active signs of tetany, but this seems to be due to the fact that merely a lesser amount of endogenous phosphate is released than in completely fasted animals. In phosphorus balance experiments this would constitute a factor of greater or lesser importance, depending upon the degree of voluntary fasting.

We should like to add that we did not wish to question the possibility of phosphate tetany from a diet high in phosphorus. From the present data it would appear that the (phosphate) tetany of fasting develops some hours later than the above, the two conditions being strikingly alike in most other respects. Dr. Shohl's statement that certain rats will die in 12 hours after ingestion of phosphate indicates that the absorption from the stomach and upper intestine may occur very suddenly and cause fatal phosphate tetany.

COMPARATIVE STUDIES OF THE METABOLISM OF AMINO ACIDS.

II. THE RATE OF ABSORPTION OF AMINO ACIDS FROM THE GASTROINTESTINAL TRACT OF THE WHITE RAT.*

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The change in view-point which followed the discovery that protein is hydrolyzed to amino acids in the intestinal tract, and is absorbed and transported in this form, has made necessary an intensive study of the behavior of the individual amino acids, components of the protein molecule, in the organism, if a true picture of protein metabolism is to be obtained. This shift in view-point has tended to simplify the study of protein metabolism since the various proteins, even those of widely different physical properties, present, when hydrolyzed, essentially the same qualitative picture. The problem thus is altered from a consideration of many compounds (the individual proteins) to a consideration of relatively few (the amino acids).

Studies of the intermediary metabolism of the individual amino acids have been concerned, for the most part, with the results of urine analysis, or in a more limited series of investigations, with the results of the analysis of the blood, following ingestion of amino acids. These have indicated the paths which may be traveled by these substances after they enter the blood stream and the end-products of the reactions of their metabolism. Time relationships, however, are indefinite. There are indications that not all amino acids are metabolized at the same rate or with equal

* The material presented here represents an abstract of part of the thesis presented by Robert Hugh Wilson in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

ease and that of the various reactions involved, some are rapid, others slow. A careful comparative study of the rates of metabolism of amino acids is still lacking. Under normal conditions of nutrition, one of the first variables of significance in the chain of events involved in the actual metabolism of the amino acids would be the rate at which each enters the blood stream from the intestine.

In previous studies by one of us (1), differences in the partition of the non-protein nitrogen of the blood, following oral administration of various amino acids, were observed in rabbits. It was suggested that the differences observed might be explained, in part, at least, by variations in the rate at which the amino acids were absorbed from the gastrointestinal canal. Direct proof of this, however, could not be obtained in the rabbit. The experimental procedure recently developed by Cori (2) has now made possible such a study of the absorption of the amino acids with the white rat. The present investigation is concerned with the rates of absorption from the gastrointestinal tract of the albino rat of the amino acids, glycine, *d*-alanine, *dl*-alanine, *d*-glutamic acid, and *l*-leucine. Further studies to include other amino acids of different types are in progress.

EXPERIMENTAL.

The method of study has been well summarized by Cori (2): "The principle is briefly as follows: A known amount of the substance under investigation is fed by stomach tube. After a given time, the rats are killed and the amount of substance remaining in the intestines is determined quantitatively. The difference between the amount fed and amount recovered from the whole intestinal tract is then the amount of substance absorbed." White rats, ranging in weight for the most part from 100 to 200 gm., were used. They were maintained on a stock diet of whole wheat bread, milk, and lettuce for periods of 7 to 30 days. At the beginning of the experiments, they were fasted for 24 hours[1] (in a

[1] Since the rats were to be used for a study of the formation of glycogen from amino acids as well as for the study of rates of absorption, the shorter fasting period of 24 hours was considered advisable since Barbour, Chaikoff, Macleod, and Orr (3) had shown that the glycogen content of white rats was lower after a 24 hour fast than after a fast of 48 hours duration. The

few cases 48 hours). The amino acids were then fed by stomach tube, the amount administered being determined by measuring an equal amount of the solution used into a 50 cc. volumetric flask and subsequently determining the amino acid nitrogen content of the solution.

After a period of 1 to 4 hours, the animals were killed either by a blow on the head or by chloroform and after ligation of the esophagus and rectum, the gastrointestinal tract was removed. This was placed immediately in an ice chamber at freezing temperature, in which it remained until the material could be analyzed. An attempt was made to keep this period, elapsing before analysis, constant (3 hours) in all experiments. Blood samples were taken from the heart immediately after the death of the animal and the amino acid content was determined colorimetrically by the method of Folin.

The gastrointestinal tract was slit open and was thoroughly washed out with about 200 cc. of hot water. To the washings were added 5 cc. of 3 per cent acetic acid, which afforded an acidity optimum for heat coagulation and flocculation of the proteins.[2] The washings were heated to boiling in order to destroy any proteolytic enzymes present and to coagulate the proteins, allowed to stand a few minutes, and filtered by suction. To the filtrate were added 5 cc. of a 10 per cent solution of sodium tungstate, enough 0.67 N sulfuric acid to cause the appearance of a flocculent precipitate,[3] and water to give a final volume of 500 cc. After 30 minutes, the precipitate was removed by filtration. To 400 cc. of the filtrate, sodium hydroxide was added until the reaction was only weakly acid to litmus, and the solution was concentrated to about 100 cc. on the steam bath. It was then made slightly alkaline with sodium hydroxide and boiled for 2 to 3 minutes to remove any ammonia present. The solution was made acid with

shorter fasting period seemed to have no effect on either the residual amino acid nitrogen of the intestine in the control animals or on the rate of absorption of glycine (*cf.* Tables I and II).

[2] The pH as measured colorimetrically in two experiments was observed to be 4.0 and 4.2.

[3] Addition of a volume of 0.67 N sulfuric acid, equal to the volume of the sodium tungstate solution added, did not result in a precipitate which could be filtered readily. Somewhat more acid was required.

acetic acid, concentrated, and made up to a final volume of 50 cc. The amino acid nitrogen of the solution was determined according to the gasometric method of Van Slyke. The procedure used in preparing the material for analysis is similar to that used by Hiller and Van Slyke (4) in the determination of amino acid and peptide nitrogen of blood, a procedure which, as shown by them, did not effect hydrolysis of peptides. It was necessary to demonstrate that quantitative recovery of added amino acid nitrogen could be obtained by the above procedure. The washings from the intestines of two rats were combined, thoroughly mixed, and divided into two equal portions. The amount of amino nitrogen present was determined in the first portion. To the second was added a known amount of leucine;[4] the proteins were then precipitated and the amino acid nitrogen determined as before. In two different experiments there was a loss in one case of 0.64 mg., and a gain of 0.46 mg. of amino nitrogen in the other, amounts which were well within the cumulative error of the three nitrogen determinations involved. Since of the amino acids studied leucine is least soluble, it is probable that similar experiments with the other amino acids would have shown recoveries equally satisfactory. These results confirm the work of Bock (5) and Hiller and Van Slyke (4), which showed that heat coagulation and tungstic acid precipitation did not precipitate the amino acids present in blood.

Residual Amino Nitrogen in the Intestine.

Before studying the rate of absorption of the amino acids, it was necessary to establish normal values for the amino nitrogen content of the gastrointestinal canal of the albino rat after short fasts. A series of control animals was treated in the same manner as were those fed the amino acids, except that the control animals received 2 cc. of distilled water and were killed shortly thereafter. The results obtained with these control rats are presented in Table I. The residual amino acid nitrogen content of the intestine was found to be rather high, a result contrasting with the value obtained for the reducing substances of the intestine by Cori (2). In a verbal communication Cori has informed us that

[4] The amounts of leucine added (140 to 180 mg.) were of the same order of magnitude as the amounts of leucine recovered from the intestine after a period of absorption in the leucine experiments.

he also has noted a fairly high residual amino acid figure, although the exact values were not given. In general it may be said that two fairly distinct groups of values were obtained; the first ten experiments recorded in Table I, which show an average value of 12.03 mg., were carried out the spring of 1928, and the remainder, designated as Series II, in the following autumn and winter. In the calculations of the amount of the amino acids remaining in the intestine after the period of absorption, this difference was taken

TABLE I.

Content of Amino Acid Nitrogen in the Intestine of Fasting Rats.

Series I.*					Series II.†				
Rat No.	Sex.	Length of fast.	Weight after fast.	Amino acid N.	Rat No.	Sex.	Length of fast.	Weight after fast.	Amino acid N.
		hrs.	*gm.*	*mg. per 100 gm.*			*hrs.*	*gm.*	*mg. per 100 gm.*
15	F.	48	187.0	14.0	63	M.	24	145.5	11.4
16	M.	48	116.0	8.6	64	"	24	92.0	6.9
17	F.	48	123.0	10.2	65	"	24	98.0	8.8
18	"	24	144.0	13.3	83	"	24	160.0	5.2
19	"	24	141.0	10.6	87	"	24	129.0	7.9
20	"	48	151.0	14.4	88	"	24	171.5	7.0
21	M.	48	194.0	15.0	89	"	24	130.5	7.6
22	"	48	193.0	10.2	99	"	24	117.0	3.8
23	"	24	94.0	10.0	123	"	24	162.0	9.7
24	"	24	108.0	14.0	124	"	24	183.5	7.5
					125	"	24	154.5	6.7
					126	"	24	163.5	6.5
Average..................				12.03	Average..................				7.42

* Determined during the spring of 1928.

† Determined during the autumn and winter of 1928–29.

into consideration, the amount subtracted being determined by the date of the experiment. Rats whose experimental number is lower than 59 belong to the same group as Series I (Table I) of the control animals.

Glycine.[5]—The rate of absorption of glycine (Table II) was determined for seventeen rats, the absorption periods ranging from

[5] The product of the laboratories of the Eastman Kodak Company.

1 to 4 hours. An inspection of Table II shows that while the average rate of absorption was 53.1 mg. of glycine per 100 gm. per

TABLE II.

Rate of Absorption of Glycine.

Rat No.	Sex.	Weight after fast.*	Absorption time.	Glycine fed.	Glycine recovered.	Rate of absorption.
		gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
9	M.	207.0	1	250	213	37
12	"	234 0	1	200	145	55
26	"	110 0	1	459	352	107
Average						66 3
10	F.	180.0	2	286	196	45
13	M.	222 0	2	208	104	52
27	"	116.0	2	440	333	54
98	"	111.5	2	315	198	59
Average						52 5
14	M.	206 0	3	221	46	58
28	"	114.0	3	452	285	56
101	"	97.0	3	362	202	53
102	F.	99.0	3	356	203	51
107	M.	166.0	3	321	194	42
108	"	156 0	3	260	124	45
Average						50 8
11	F.	172.0	4	315	116	50
100	M.	109.5	4	321	124	49
109	"	127.0	4	420	222	50
110	"	115.5	4	351	190	40
Average						47.3
" of all experiments						53.1
" " 2. 3. and 4 hr. experiments						50.3

* Rats 9 to 14 were fasted for 48 hours, the others 24 hours.

hour, the values ranged from 37 to 107 mg. If, however, the results obtained with the 1 hour absorption period are omitted, the average becomes 50.3 mg., with variations of only 40 to 58 mg.

per 100 gm. per hour. As will be shown later, a much greater variability would be expected during the shorter period, and for this reason the average value for the 2, 3, and 4 hour absorption periods is probably more nearly the true value.

At this point in order to make Tables II to VIII clear it may be desirable to illustrate the procedure by citing the details of a typical experiment. Rat 10 (Table II), a female weighing 180 gm., was fed a glycine solution containing 103.3 mg. (uncorrected) of amino acid nitrogen as determined by the Van Slyke method. Since this method gives values which are toò high when glycine is used, this figure has to be corrected by multiplying by 0.93. As glycine contains 18.67 per cent nitrogen, the amount of glycine fed was $\frac{103.3 \times 0.93}{0.1867}$ = 515 mg., or 286 mg. per 100 gm. The intestinal contents contained 92.6 mg. of amino acid nitrogen, or 51.4 mg. per 100 gm. The experiment was carried out in February, 1928. Therefore the blank for the spring series, 12.03 (Table I), was subtracted from this, leaving 39.4 mg. of amino acid nitrogen due to the glycine fed or 196 mg. of glycine per 100 gm. (the factor 0.93 again being taken into consideration). The difference between the amount fed, 286 mg., and the amount remaining in the intestinal tract is the amount absorbed (90 mg.). Since the absorption took place over a 2 hour period, the rate of absorption was 45 mg. per 100 gm. per hour.

If we consider now the variations in rate of absorption during the 1 hour period, the reason, at least in part, for these can be seen. On the assumption that a certain rat has a residual nitrogen content of 8.6 mg. instead of 12.0 mg. per 100 gm., which has been shown in Table I to be quite possible, the amount of glycine not absorbed will actually be 16.9 mg. more than the value just computed. With only a 1 hour absorption period, this error would be transmitted bodily to the absorption rate column, while if the period had been longer, this error would have been divided by the number of hours, so that the determinations over the longer periods should show a greater similarity, as has been shown to be true. This variability due to unlike residual nitrogen contents, would be even greater when the nitrogen content of the amino acid fed is less than that of glycine, and for this reason, in some of the absorption experiments to be reported, the 1 hour absorption period has been eliminated entirely

On the other hand, a determination for a period longer than 4 hours is not practicable because of the difficulty in obtaining a solution concentrated enough to allow absorption to proceed for a greater length of time, and because with the larger doses and particularly when the amino acid is fed as a salt, diarrhea often develops in a few hours. While, to a large extent, the variations in the rate of absorption of the shorter periods can be explained by the above reasoning, this is not entirely true, some of the values being outside of this range of variability.

Glycine as the Sodium Salt.—Because leucine and glutamic acid, two of the amino acids which were used for determinations of the rate of absorption, were too insoluble to make a solution as concentrated as was needed for this study when in an uncombined state, they were fed as sodium salts. It seemed possible that the introduction of the sodium ion might change the absorption rate of these acids, so, in order to see if this were the case, glycine to which had been added approximately one-half of the amount of sodium hydroxide needed to form the sodium salt, was fed. Only half of the glycine was made into the salt because the amount of sodium hydroxide necessary to accomplish this is about equal to that required to form the sodium salt of leucine and the monosodium salt of glutamic acid. While the ionization and the osmotic pressure of these solutions of the sodium salt of glycine probably differed from those of the sodium salts of leucine or of glutamic acid, it was felt that the way in which the salt tended to act might be determined.

The results of this experiment are shown in Table III. The rate of absorption is clearly higher than for glycine, and, with the exception of the 1 hour period, quite constant, the average being 64.2 mg. as compared with 50.3 mg. per 100 gm. per hour, the value found for glycine.

dl-Alanine.[6]—*dl*-Alanine was fed to fourteen rats, the time allowed for absorption ranging from 2 to 4 hours. As will be seen in Table IV, the absorption rate found was considerably higher than was the case with glycine; the average value was 73.6 mg. per 100 gm. per hour, with values varying from 45 to 92 mg., the determinations being much less constant than those obtained

[6] Obtained from the Eastman Kodak Company and the Pfanstiehl Company.

with glycine or its salt. In spite of this variation, the values, with only two exceptions, are higher than the highest value found for glycine in a 2 to 4 hour absorption period.

d-Alanine.[7]—Because of the high cost of this material an extended series of experiments was not possible. However, we were

TABLE III.

Rate of Absorption of Glycine Fed as the Sodium Salt.

Rat No.	Sex.	Weight after fast.	Absorption time.	Glycine fed.	Glycine recovered.	Rate of absorption.
		gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
39	M.	170.0	1	187	102	85
42	"	187.0	1	152	73	79
49	"	227.0	1	158	118	40
Average						68.0
41	F.	152.0	2	192	65	64
43	"	143.0	2	199	83	58
47	M.	202.5	2	177	59	59
48	"	203.5	2	179	52	64
111	"	117.0	2	489	344	73
Average						63.6
112	M.	137.5	3	416	224	64
114	"	141.0	3	405	219	62
Average						63.0
113	M.	148.0	4	386	155	58
Average of all experiments						64.2
" " 2, 3, and 4 hr. experiments						62.8

able to determine the rate of absorption of this acid over a 3 hour period with six rats. As will be seen in Table IV, with one exception, the determinations were very uniform, the rate of absorption being found to be practically the same as that of the racemic acid.

[7] Obtained from the Hoffmann-LaRoche Company.

TABLE IV.

Rate of Absorption of dl-Alanine and d-Alanine.

Rat No.	Sex.	Weight after fast.	Absorption time	Alanine fed.	Alanine recovered.	Rate of absorption.
			dl-Alanine.			
		gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
67	M.	128.5	2	233	65	84
70	"	146 0	2	364	249	58
94	"	109.0	2	352	169	92
96	"	117.5	2	323	173	75
Average						77 3
66	M.	108 5	3	276	28	83
68	"	109 5	3	287	96	64
71	"	134.0	3	394	259	45
72	"	100.5	3	529	263	89
95	"	115 0	3	334	134	67
97	"	107.0	3	355	107	83
116	"	149 0	3	271	47	75
118	"	162 0	3	245	42	68
Average						71.8
115	M.	129 0	4	312	10	76
117	"	123 5	4	321	36	71
Average						73 5
" of all experiments						73 6
			d-Alanine.			
157	M.	104 5	3	303	30	91
159	"	113.5	3	279	43	79
160	"	108.0	3	294	70	75
161	"	130.5	3	290	68	74
162	"	133.5	3	284	73	70
163	"	128.5	3	295	63	77
Average						77.7

dl-Alanine as the Sodium Salt.—For the reason already discussed, *dl*-alanine to which half of the theoretical amount of sodium hydroxide had been added was fed. Of the fifteen rats

used, seven had to be discarded, as alanine, when fed as the salt, produced a marked diarrhea in a short time. For the same reason all of the values which were obtained were for a fairly short period, only two experiments being successful for a 3 hour period. The average rate of absorption (Table V) was found to be a value decidedly lower than was found for *dl*-alanine, in marked contrast to the rise in absorption rate when the sodium salt of glycine was fed in place of glycine.

TABLE V.

Rate of Absorption of dl-Alanine Fed as the Sodium Salt.

Rat No.	Sex.	Weight after fast.	Absorption time.	Alanine fed.	Alanine recovered.	Rate of absorption.
		gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
143	M.	128 0	2	301	224	39
146	F.	138 0	2	277	176	51
153	M.	146 0	2	271	164	54
164	"	174 0	2	212	115	48
166	"	192 0	2	195	132	32
167	"	138 5	2	264	171	47
Average						45 2
140	M.	140 5	3	341	190	50
144	"	117 0	3	329	181	49
Average						49.5
" of all experiments						46.3

l-Leucine.[8]—*l*-Leucine was fed as the sodium salt for absorption periods of 2 to 4 hours. Although there was considerable variation noticed (Table VI), the results are unquestionably lower than those found for the other amino acids studied, the values ranging from 25 to 57 mg., with an average of 42 mg. of leucine absorbed per 100 gm. per hour.

d-Glutamic Acid.[9]—The feeding of the monosodium salt of *d*-

[8] The leucine (Pfanstiehl) dissolved in 20 per cent hydrochloric acid showed a specific rotation of +20.3° as compared with the value of +15.8° obtained by Fischer and Warburg (6).

[9] The glutamic acid was prepared from Ajinomoto in this laboratory.

glutamic acid gave varying rates of absorption (Table VII). Since this amino acid has a rather high molecular weight and a low nitrogen content, a slight variation in the residual nitrogen of the intestinal tract would produce a considerable change in the calculated absorption rate. The values are so high in some instances, however, that it is very doubtful whether this can be the complete explanation. The small number of experiments for the

TABLE VI.

Rate of Absorption of l-Leucine Fed as the Sodium Salt.

Rat No.	Sex.	Weight after fast.	Absorption time.	Leucine fed.	Leucine recovered.	Rate of absorption.
		gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
78	M.	164.0	2	192	143	25
81	"	175.5	2	183	88	48
82	"	174.0	2	166	72	47
Average						40.0
79	M.	111.5	3	288	116	57
80	"	152.0	3	207	105	34
84	"	158.0	3	182	63	40
103	"	134.0	3	234	126	36
105	"	100.5	3	313	165	49
Average						43.2
104	F.	111.5	4	282	126	39
106	M.	115.5	4	273	89	46
Average						42.5
" of all experiments						42.1

shorter periods of absorption makes it impossible to reach any definite conclusion regarding the absorption rate immediately after the ingestion of glutamic acid, but it appears that after an initial very rapid absorption, there is a very decided drop so that according to the averages in Table VII, the absorption has decreased from 102.3 mg. per 100 gm. during the 1st hour to 8.7 mg. between the 2nd and 3rd hours, with an increase again to 76.9 mg. between the 3rd and 4th hours. However, the wide variations

found during the first 2 hours make the average value for these periods of very little significance. While it is doubtful if there

TABLE VII.

Rate of Absorption of d-Glutamic Acid Fed as the Monosodium Salt.

Rat No.	Sex.	Weight after fast.	Absorption time.	Glutamic acid fed.	Glutamic acid recovered.	Rate of absorption.
		gm.	*hrs.*	*mg. per 100 gm.*	*mg. per 100 gm.*	*mg. per 100 gm. per hr.*
30	M.	187.0	1½	264	187	84
51	F.	169.5	1	360	244	116
52	"	168.5	1	361	254	107
Average						102.3
34	M.	179.5	2	277	163	57
35	"	162.0	2	307	136	85
50	F.	146.5	2	416	222	97
55	M.	224.5	2	285	78	104
Average						85.8
31	M.	170.0	3	293	135	53
33	"	143.0	3	344	149	65
54	F.	173.0	3	370	195	58
127	M.	105.0	3	487	265	74
130	"	167.5	3	308	125	61
132	"	126.0	3	326	157	56
133	"	167.5	3	243	81	54
Average						60.1
128	M.	132.0	4	390	100	73
129	"	150.5	4	340	107	58
131	"	129.0	4	319	70	62
134	"	138.0	4	295	40	64
Average						64.3
" of all experiments						73.8
" " 3 and 4 hr. experiments						61.6

is as extreme a drop as this with a subsequent increase, it does seem as if there may be a greater initial rate than is found later.

TABLE VIII.

Rate of Absorption of Amino Acids. A Comparison of the Methods of Calculation on the Basis of Unit of Body Weight and Unit of Body Surface.

Substance fed.	Absorption period.	No. of experiments.	Absorption.					
			Amino acid.	N	Variation.	Amino acid.	N	Variation.
	hrs.		*mg. per 100 gm. per hr.*	*mg. per 100 gm. per hr.*	*per cent*	*mg. per 100 sq. cm. per hr.*	*mg. per 100 sq. cm. per hr.*	*per cent*
Glycine.	1	3	66.37±22.30	12.39	33.6	36.80±12.44	6.87	33.8
	2	4	52.28± 2.87	9.76	5.5	30.05± 1.48	5.61	4.9
	3	6	50.92± 3 54	9.51	7.0	28.80± 2.29	5.38	8.0
	4	4	47.15± 2 67	8.80	5.7	26.38± 1.81	4.93	6.9
	1–4	17	53.08± 5.52	9 91	10 4	29 94± 3.41	5.59	11.4
	2–4	14	50.23± 3.06	9 38	6.1	28.46± 1.97	5.31	6.9
Glycine (Na salt).	1	3	68 17±15 46	12 73	22.7	39 30± 9.91	7.34	25.2
	2	5	63.40± 2 78	11 84	4.4	37.02± 1.38	6.91	3.7
	3	2	63.00± 0 96	11 76	1.5	35.95± 0.43	6.71	1.2
	4	1	57 80	10.79		33.50	6.25	
	1–4	11	64.12± 5.72	11.97	8.9	37.13± 3.44	6.93	9.3
	2–4	8	62.60± 2 34	11.69	3.7	36.31± 1.18	6.78	3.3
dl-Alanine.	2	4	77.22± 8.31	12.14	10.8	42.78± 4.23	6.73	9.9
	3	8	71.43± 7.73	11.23	10.9	39.19± 3.90	6.17	9.9
	4	2	73.35± 2.15	11.54	2.9	40.70± 1.43	6.40	3.5
	2–4	14	73.36± 6.83	11 55	9.3	40.43± 3.41	6.35	8 4
d-Alanine.	3	6	77.70± 3.60	12 22	4.6	42.43± 1.60	6.67	3.8
dl-Alanine (Na salt).	2	6	44 87± 4.88	7.06	10.9	25.87± 3.58	4.07	13.8
	3	2	49.85± 0.33	7.85	0 7	27.95± 0.72	4.40	2.6
	2–3	8	46.11± 4.03	7.25	8.7	26.39± 2.26	4.15	8.6
l-Leucine (Na salt).	2	3	39.70± 8.32	4.24	21.0	22.97± 5.05	2.46	22.0
	3	5	43.30± 6.06	4.63	14.0	24.24± 2.84	2.58	11.7
	4	2	42.35± 3.29	4.53	7.6	23.20± 1.79	2.48	7.7
	2–4	10	42.03± 5.24	4.49	12.5	23.65± 2.82	2.53	11.9
d-Glutamic acid (Na salt).	1	3	102.67±10.00	9.78	9.8	58.13± 6.63	5.53	11.4
	2	4	85.55±11.33	8.15	15.6	50.38± 7.22	4.80	14.4
	3	7	60.19± 4.05	5.73	6.7	34.50± 1.92	3.28	5.6
	4	4	64.10± 3.27	6.10	5.1	36.40± 1.71	3.47	4.7
	1–4	18	73.77±11.63	7.03	15.8	42.39± 6.87	4.04	16.2
	3–4	11	61.61± 3.76	5.86	6.1	36.19± 1.85	3.35	5.3

Body Surface in Relation to Rate of Absorption.

Pierce, Osgood, and Polansky (7) computed the rate of absorption of glucose on the basis of unit of body surface as well as unit of body weight. While their results did not indicate any notable advantage of either method of calculation in the matter of uniformity, they considered them slightly better when calculated on the basis of body surface. It was accordingly decided to calculate in both ways in this study to see if any more definite conclusion could be reached than was obtained by the above authors.

For the computation of body surface, the usual formula of Meeh, $S = kW^{\frac{2}{3}}$, was used. Benedict and MacLeod (8) have recently reviewed the literature concerning the value of k for the white rat and have concluded that the older Rubner constant (9.1) is most nearly correct,[10] a conclusion supported by the more recent direct measurements of Lee (9). We have used the Rubner constant in our own study.

A duplication of the preceding tables, changed only in respect to the basis of calculation, is hardly necessary. In Table VIII a summary of the rates of absorption of the amino acids studied is presented, the rates being calculated in the two ways and the percentage variations compared. A study of Table VIII shows that neither method of calculation has much of an advantage over the other in the case of rats, confirming the findings of Pierce, Osgood, and Polansky (7) for carbohydrates. Table VIII contains in addition to the above, the rates of absorption of the amino acids as calculated on the basis of nitrogen absorbed, thus giving a comparison of the number of molecules of the acid absorbed per unit of time. On this basis the amino acids can be arranged in the following order of decreasing rates of absorption: *d*-alanine, glycine (Na), *dl*-alanine, glycine, *dl*-alanine (Na), *d*-glutamic acid (Na), and *l*-leucine (Na).

DISCUSSION.

A general survey of the work of other investigators indicates that there exists a considerable difference in the rates of absorption and catabolism of the various amino acids. There is quite general agreement that glycine and alanine are absorbed more

[10] Benedict and MacLeod (8), pp. 360–361.

rapidly than the other acids (Folin and Denis (10), Levene and Kober (11), Levene and Meyer (12), Seth and Luck (13), Johnston and Lewis (1)). It should be remembered, however that all of the evidence is indirect, being based on blood and urine analyses, and this involves a second variable, the ease of disposal of the amino acids by the tissues. That the methods used can be said to give a fairly reliable picture of relative rates of absorption has been demonstrated in this paper, since the results obtained here (*on the basis of nitrogen*, the basis on which all of the former work was done) show that the rates of absorption of glycine and alanine are clearly greater than those of leucine and glutamic acid.

We are unable to agree with Bang (14) concerning the rate of absorption of leucine. While he found no increase in the rest nitrogen of the blood within 10 hours after feeding leucine, he did report an increase in blood urea nitrogen of 12 to 14 mg. per 100 cc. indicating a fairly rapid absorption with immediate deamination. The work of Seth and Luck has also shown a small increase only in the amino acid nitrogen, but it did not show a marked rise in urea nitrogen, their maximum rise in this blood constituent being less than 5 mg. per 100 cc., which indicates a slower absorption such as we have shown actually exists in rats.

The most serious disagreement between this work and that of other investigators is in the rate of absorption of alanine. Cori (15) reported a series of experiments in which he found the rate of absorption of glycine to be 0.048 gm. and of alanine, 0.045 gm. per 100 gm. per hour.[11] While the absorption rate of glycine as found by us (50 mg. per 100 gm. per hour) is in agreement with that given by him, we find a distinctly higher rate for alanine (73 mg. per 100 gm. per hour), a rate which is greater than that of glycine not only when considered in terms of the acid absorbed, but even when expressed in terms of nitrogen. As was indicated in the experimental part, there was considerable variation noted in the rate of absorption of alanine by different animals. However, of the fourteen rats used, only one had a rate as low as the

[11] Cori's paper is a preliminary note and full details of his work are not given. If he had used the sodium salt of alanine (as he may have done, although from the wording of the paper this hardly seems probable) his results for alanine (45 mg. per 100 gm. per hour) would agree with ours.

average reported by Cori, and only two rates were lower than the highest obtained for glycine in a 2 to 4 hour absorption period.

This higher rate of absorption explains as well as, or perhaps better than, the lower value, some of the findings previously observed. While the amino acid nitrogen of the blood rises sharply whenever glycine or alanine is fed, the urea nitrogen figures sometimes show a difference. Seth and Luck (13) in experiments with rabbits, recorded the amino acid and urea nitrogen values of the blood after the ingestion of amino acids. The sum of the rises in the amino acid and the urea nitrogen values was slightly greater after alanine than after glycine ingestion. In the work presented by Johnston and Lewis (1), the sum of these rises over the first 3 hours after the feeding of glycine was about the same as was found after alanine. Since alanine has a lower content of nitrogen than has glycine, equal changes in nitrogen would indicate a greater absorption of alanine. On the other hand, Johnston and Lewis found a greater rise in the non-protein nitrogen after glycine than after alanine, a finding which suggests a slower absorption of the alanine nitrogen. Seth and Luck also found that neither the urea nitrogen nor the amino acid nitrogen of the blood rose as much after the injection of alanine into an intestinal loop of a dog as was observed when glycine was used.

In all studies involving changes in the nitrogen content of the blood, it must be borne in mind that the magnitude of these changes is determined not only by the rate of absorption from the intestine, but also by the rate or removal from, and addition to, the blood of nitrogenous substances by the tissues. Luck (16) found that the amino acid nitrogen content of the liver and muscles of a rat was not as great after alanine ingestion as it was after glycine. This would suggest either that the alanine was less readily absorbed by the tissues or that it was catabolized more rapidly. Adequate studies of the urea content of tissues after the ingestion of amino acids have not been made. The observations made by Johnston and Lewis led them to believe that glycine may have been deaminized more slowly than alanine.

The position of glutamic acid as intermediate, in the rate of its absorption (on the basis of equivalent amounts of nitrogen), between glycine and alanine on the one hand and leucine on the

other, is in agreement with the blood analysis experiments of other investigators. Johnston and Lewis concluded that in the rabbit the rate of absorption of glutamic acid was probably less than that of glycine or of alanine. Seth and Luck observed that while there was little, if any, rise in the amino acid content of the blood following the ingestion of glutamic acid, there was a steady and significant increase in the urea nitrogen, a rise not found after leucine feeding. These results suggest that the rate of absorption, in terms of nitrogen, of glutamic acid is less than that of glycine or of alanine and greater than that of leucine, a condition which we have shown to exist in the rat, at least for the longer absorption periods. Csonka (17) fed isoglucogenic quantities of glycine, alanine, and glucose to phlorhizinized dogs. His conclusion was that "the rapidity of the absorption and elimination of glucose ingested in phlorhizin glycosuria is almost the same as the rapidity of the absorption, deamination, synthetic sugar production, and the elimination of such sugar, after ingestion of isoglucogenic quantities of glycocoll or alanine." If glucose was excreted by these animals as soon as it entered the blood, the conclusion could be drawn that the time required for the absorption of the amino acids and the sugar formation from them must have been of the same order as the time required for the absorption of the glucose.

Cori (2, 18) found that glucose was absorbed by rats at a rate of about 0.2 gm. per hour. In rats, then, the rate of absorption of glucose is several times as rapid as is the rate of absorption of amino acids. There are several possible explanations which may explain the lack of correlation of these results with those observed by Csonka.

It is possible that the excretion of ingested glucose does not occur as soon as it is absorbed. If this were the case, the absorption and conversion of the amino acids to glucose might occur during the period of retention of the ingested glucose. It was shown by Csonka, however, that the excretion of the ingested glucose began almost immediately after ingestion, a fact that does not suggest a retention of any considerable amount.

It must always be remembered that observations obtained on one species of animal must be applied with caution to other species. It may therefore be possible that the rates of absorption

of glycine and alanine by the dog are similar to that of glucose. While this would be very different from findings with rats, it still exists as a possibility.

A third explanation of the equal rates of excretion of extra glucose after glycine, alanine, and glucose ingestion is that the extra glucose after amino acid administration may be in part due to a stimulation leading to a depletion of the sugar already present in the body rather than to a conversion of the amino acid to sugar. In this regard, it is interesting to note that those acids which stimulate metabolism to the greatest extent are, in many cases, the sugar-forming acids. There are, however, several strong arguments against this explanation of excretion of extra glucose because of a stimulation by the ingested amino acids. The amino acid which has the greatest specific dynamic action (phenylalanine) does not cause an excretion of extra sugar by the diabetic organism. A further argument against this explanation is that these amino acids lead to the excretion of a very uniform amount of extra glucose, an amount exactly corresponding to all of the carbon atoms in glycine and alanine.

One other possible explanation exists, one that, as far as we have found, has no evidence for or against it. The rats used for the absorption experiments were normal animals; the dogs used by Csonka were not. Is it possible that phlorhizin will so alter the intestinal wall as to make the rates of absorption of these two amino acids similar to that of glucose? If this were so, the results obtained by Csonka could be easily explained.

The statement made by Cori (15) that the absorption of glycine and alanine could be represented by a straight line curve has been borne out by the experiments presented here, although perhaps with reservations. It will be noticed by referring to Table VIII that, with the exception of glutamic acid, the hourly rates for each substance studied are much the same, whether the absorption period be of 2, 3, or 4 hours duration, but that there appears in the studies of most of the acids a slight gradual decrease in the rate with the time. This would indicate the possibility that a marked decrease in the absolute amount of the amino acid present in the intestine may cause a slight decrease in the rate at which it can be absorbed. In order to say definitely that such a decrease with longer periods of time actually exists, the number of experiments

would have to be greatly increased, as the change is apparently not great and the individual determinations overlap. The study would have to be a statistical one.

Again, in the more detailed tables it will be seen that often, although not always, there is a higher rate of absorption observed with the animals given the greatest amount of amino acid. So while there seems to be a tendency for a uniform absorption regardless of the absolute amount of substance present in the gastrointestinal tract, it would appear that the absolute amount may exert some influence. It is evident from some recent work of Cori and Cori (19), that the rule of uniform absorption may not be applicable generally, since they observed with lactic acid and sodium lactate that the rate of absorption was greatly influenced by the amount fed.

In the experimental part it was mentioned that it was necessary to feed glutamic acid and leucine as salts. For this reason determinations were made of the rates of absorption of glycine and of alanine as their sodium salts so as to get some idea as to what differences might be expected because of the presence of the sodium ion. The results were found to be contradictory and the question is as far from being answered as before, except that it can be definitely stated that the amino acids, when fed as their sodium salts, may be absorbed at a rate quite different from that observed when they are fed as the free acids.

Cori and Cori (19) suggested that the greater absorption rate which they found when sodium lactate rather than lactic acid was fed, might be explained by the lower acidity of the sodium lactate. Since the addition of sodium hydroxide to alanine caused a decrease in its rate of absorption, this explanation could certainly not be applied here. That the much slower absorption of alanine as its sodium salt may be due to a certain toxic action of the compound is possible, as an acute diarrhea developed in so many of the rats, sometimes in a very short time. One rat, for instance, had a severe diarrhea in 15 to 30 minutes after being fed the alanine salt. An argument against the toxicity of this compound is that those animals which did not develop the diarrhea were fed as much as the others.

SUMMARY.

1. The rates of absorption from the intestinal canal of rats, of glycine, *dl*- and *d*-alanine, when fed as the free acids, and of glycine, *dl*-alanine, *d*-glutamic acid, and *l*-leucine when fed as the sodium salts, have been determined. The amino acids can be arranged in the following descending order of rate of absorption: *d*-alanine, *dl*-alanine, glycine (Na), *d*-glutamic acid (Na), glycine, *dl*-alanine (Na), and *l*-leucine (Na). In terms of equivalent amounts of nitrogen, the order is as follows: *d*-alanine, glycine (Na), *dl*-alanine, glycine, *dl*-alanine (Na), *d*-glutamic acid (Na), and *d*-leucine (Na).

2. The statement made by Cori that the rate of absorption is independent of the absolute amount and the concentration of the amino acid in the intestine, has been confirmed.

3. It has been shown that changes in the nitrogenous constituents of the blood after the ingestion of an amino acid can be partially explained by a consideration of the rate of absorption of the acid.

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NOTE ON THE PREPARATION OF THE MONOAMINO ACIDS FROM THEIR PICRATES.

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INTRODUCTION.

The usual method for obtaining the free amino acids from their picrates is by decomposition by means of an excess of sulfuric acid, extraction of the picric acid with ether or hot benzene, exact removal of the sulfate ion with barium hydroxide and, following the filtration and washing of the barium sulfate, evaporation of the aqueous solution. We have found that aniline, being a stronger base than the monoamino acids, will decompose their picrates, and, if an excess is used, it will dissolve the aniline picrate formed and make possible its removal from the aqueous solution of the amino acid.

We have successfully applied aniline to the decomposition of the picrates of proline and glycocoll and have in each case very readily obtained the free amino acids in larger yields than by the older method. Town (1) has described a method of preparing proline which requires its purification as the picrate. We have attempted to prepare proline as the picrate following extraction of hydrolyzed gelatin with alcohol and succeeded in obtaining yields equal to those that we secured using Town's method. We encountered difficulty in the very slow rate of filtration of the extractions and recommend the procedure of Town, supplemented by our method of preparation of the free proline.

We have frequently prepared glycocoll picrate as a by-product in the preparation of amino acids from gelatin. The picrate may possibly be employed as a means of isolating glycocoll obtained by synthetic methods. Since, therefore, the picrate is a practical salt of glycocoll, we have applied our method to it for the recovery of the free amino acid.

The basic dissociation constants of proline (2), glycocoll (2), and aniline (3) are as follows:

Proline $K_b = 1 \times 10^{-12}$
Glycocoll $K_b = 2.6 \times 10^{-12}$
Aniline $K_b = 4.6 \times 10^{-12}$

EXPERIMENTAL.

Proline.—Suspend 80 gm. of proline picrate (melting at 150–151°, corrected) in 600 cc. of water in a 2 liter separatory funnel. Shake it with 200 cc. of aniline until the picrate is decomposed and draw off the aniline layer. Repeat the extraction with a second 200 cc. portion of aniline. Wash the combined aniline extracts with 500 cc. of water and add the washings to the main aqueous solution. Extract the proline solution with four 200 cc. portions of ether. Boil the solution with 30 gm. of norit until it is entirely colorless. Concentrate the filtrate from the norit almost to dryness *in vacuo*. Dissolve the proline by warming with 500 cc. of absolute alcohol. Cool, filter off any insoluble material, and add dry ether until no further precipitation occurs (300 to 600 cc.). Set the mixture in an ice box overnight to complete the precipitation. Filter off the proline and wash it with a small amount of dry ether. Dry it in air or in a vacuum oven at 60°.

The yield of pure *l*-proline is 24 to 25 gm. or 90 to 94 per cent of the theoretical. Its melting point is 214–215°, corrected. It is free of primary amino nitrogen.

Glycocoll.—Suspend 50 gm. of glycocoll picrate (melting point 198–199°) in 500 cc. of water and treat exactly as in the procedure for proline. Evaporate the aqueous solution obtained after decoloration with norit to about 100 cc. Add 300 cc. of 95 per cent ethyl alcohol and set in the ice box overnight. Filter off the glycocoll, wash it with 95 per cent alcohol, and dry it in air. The yield is 18 gm. of pure white glycocoll, or about 90 per cent of the theoretical.

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ARGININE FEEDING AND CREATINE-CREATININE EXCRETION IN MAN.

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Biochemical literature records numerous attempts to discover the origin of creatine and creatinine. Such experiments usually have involved the feeding or injection of substances known to occur in the body, or of synthetic products related in chemical structure to creatine or its anhydride. Most of these investigations have yielded negative results. The chief exceptions have been observed in connection with experiments involving the administration of arginine. Inasmuch as this amino acid is the only component of the protein structure known to contain the guanidine group, attention naturally was directed toward it as the most likely source of creatine. According to Thompson (1917,*a*) the administration of arginine carbonate, either orally or parenterally, leads to an increased output of total creatinine in the urine. The increase is said to be more pronounced when paraformaldehyde or hexamethylenetetramine is furnished along with the arginine as a possible source of formaldehyde for the methylation reaction involved in the synthesis of creatine from arginine (Thompson, 1917,*b*). Furthermore, the same investigator (1917,*a*) reports that the intramuscular administration of arginine results in the utilization for creatine formation, of approximately 14.5 per cent of the guanidine present in the amino acid.

Other experiments leading to similar conclusions are those of Inouye (1912) and Jansen (1917). The former finds that the surviving liver produces creatine when perfused with an arginine solution, while the latter states that increased muscle tonus is accompanied by the disappearance of arginine and the formation of an equivalent amount of creatine. Perhaps the most striking evidence for the origin of creatine in arginine is to be found in the experiments of Gross and Steenbock (1921). These investigators report that the oral administration of arginine to pigs on a nitrogen-free diet leads to pronounced increases in the output of creatine in the urine. The dose of arginine ordinarily employed was 4.08 gm., which was taken as the arginine content of 100 gm. of casein. The authors point out that generally about 25 per cent more creatine was excreted after casein

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* On leave of absence from Wells College.

feeding than after the administration of the arginine equivalent of the protein. They are inclined to the belief that the greater effect of casein may be explained on the basis of a stimulation of the endogenous metabolism by the phosphoric acid liberated from the protein. In line with this suggestion they state that the creatinuria of cystine feeding, observed first by Harding and Young (1920), occurs only when the sulfuric acid formed by the oxidation of the amino acid is left unneutralized.

In contrast to the findings outlined above, many investigators have been unable to show any correlation between the arginine intake and the creatine-creatinine output in the urine, or the creatine content of the tissues. Thus Van Hoogenhuyze and Verploegh (1905) observed no alteration in the excretion of creatinine following the administration of gelatin, a protein relatively rich in arginine. Jaffé (1906) found no increase in urinary creatinine as a result of arginine injections into rabbits. Myers and Fine (1915) were unable to detect significant differences between the creatine content of the muscles of rats fed diets containing edestin with 14 per cent of arginine, and casein with 4 per cent of the amino acid. Baumann and Marker (1915) by means of the perfusion technique, and Baumann and Hines (1918) as the result of injection experiments, discovered no relationship between arginine and creatine in dogs. Rose and Cook (1925) observed a similar lack of correlation between the arginine content of the diet and the output of total creatinine in rats. It is evident, therefore, that the available data are exceedingly contradictory.

The observations of Benedict and Osterberg (1923) upon dogs, and of Rose, Ellis, and Helming (1928) upon man that the oral administration of constant quantities of creatine leads to a very gradual increase in creatinine excretion which may not attain a maximum until after the lapse of several weeks, suggested the possibility that the synthesis of creatine from arginine might also be a very slow process. If creatine is one of the *catabolic* products arising in the utilization of exogenous arginine, then the ingestion of moderate doses of the amino acid over long periods of time might be expected to lead to increases in the output of creatine, creatinine, or both. On the other hand, if creatine is an *anabolic* product, the synthesis of which is limited quantitatively to the needs of the organism for it, the administration of arginine in excess of the anabolic requirements would not be expected necessarily to exaggerate creatine-creatinine excretion. With these considerations in mind two prolonged experiments were conducted. As in the creatine feeding experiments of Rose, Ellis, and Helming, one subject was a male and the other a female. Indeed, the latter was the same individual in the two investiga-

tions. Both subjects were normal graduate students, and were selected for the purpose in question because of their similarity in body weight. The diets, the composition of which is shown in Table I, were identical with those employed by Rose, Ellis, and Helming. As will be observed, the male subject ingested a slightly larger quantity of potatoes than did the female. In spite of this fact the relatively high nitrogen output and moderate

TABLE I.

Composition of Diets.

	Male subject.	Female subject.
	gm.	*gm.*
Bread (whole wheat)	200	200
Butter	75	75
Eggs (raw weight)	100	100
Milk	1200	1200
Potato (boiled)	200	150
Beans (canned Lima, cooked)	125	125
Lettuce	100	100
Mayonnaise	20	20
Bran	25	25
Sugar	15	15
Jelly	40	40
Orange	300	300
Nitrogen content,* *gm.*	14.75	14.55
Energy value,* *calories*	2970†	2920

* Most of the nitrogen and energy data are taken from Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric.*, *Office Exp. Stations*, *Bull. 28* (revised) (1906); but the milk, mayonnaise, jelly, beans, and bran were analyzed for total nitrogen.

† Beginning with the third period of arginine administration the sugar content of the diet of the male subject was increased to 65 gm. daily. Thus the energy intake was raised to approximately 3170 calories.

hunger experienced by the male indicated that the calorific intake was not quite adequate. Beginning, therefore, with the 3rd week of arginine feeding the sugar content of the diet was increased from 15 to 65 gm. daily. Part of this was consumed in the form of lump sugar between meals. Even with this addition to the ration nitrogen and calorific equilibrium probably were just about maintained. The young man was a very active individual, and was

engaged in a research which required his attention not only throughout the day but usually in the evenings as well.

The arginine monochloride was prepared in this laboratory and was of known purity. The daily dose for each individual was 1.606 gm., equivalent to 1 gm. of creatine. This was mixed with 0.64 gm. of sodium bicarbonate, equivalent to the hydrochloric acid present in the amino acid salt, and administered in two equal doses, dissolved in milk, at the morning and noon meals.

TABLE II.

*Arginine Feeding and Creatine-Creatinine Excretion.**

Subject 1, Male.

Days.	Body weight.	Urine.			Creatinine coefficient.	Remarks.
		Total N.	Creatinine.	Creatine as creatinine.		
	kg.	*gm.*	*gm.*	*gm.*		
1–7	70 5	12.81	1.86	0.01	26.4	Fore periods without added arginine.
8–14	69.7	13.65	1.84	0	26.4	
15–21	70.0	14.28	1.84	0	26.3	Periods of arginine administration. 1.606 gm. arginine monochloride and 0.64 gm. sodium bicarbonate administered daily in two equal doses.
22–28	69.2	14.12	1.81	0	26.2	
29–35	69 1	14.57†	1.81	0	26 2	
36–42	68.6	13.65	1.79	0	26 1	
43–49	69 1	13.27	1.71	0 06	24.7	
50–56	68 6	13.10	1.77	0	25.8	
57–63	68.6	12 66	1 71	0.01	24.9	After periods without added arginine.
64–70	68 6	13.01	1.70	0	24 8	

* The figures represent daily averages for the periods indicated.

† Beginning with this period the sugar content of the diet was increased from 15 to 65 gm.

Great care was exercised in the urine analyses to insure the maintenance of identical conditions throughout. The urines were diluted to the same volumes each day preliminary to the analyses. Total nitrogen was determined by the Kjeldahl-Gunning method, and total and preformed creatinine were estimated according to the procedures of Folin (1914). Highly purified creatinine zinc chloride served as the standard. The subjects consumed the constant diets for 17 days preceding the urine collections. At no time did

the urines contain protein, sugar, or other recognized abnormal components.

The results of the two experiments are outlined in Tables II and III. Inasmuch as the arginine feeding exerted no detectable influence upon either the creatine or creatinine output, it has seemed unnecessary to reproduce the results of the daily analyses. The data in Tables II and III represent the average daily output of each component for the period in question. All periods were of

TABLE III.

*Arginine Feeding and Creatine-Creatinine Excretion.**

Subject 2, Female.

Days.	Body weight.	Urine.			Creatinine coefficient.	Remarks.
		Total N.	Creatinine.	Creatine as creatinine.		
	kg.	*gm.*	*gm.*	*gm.*		
1–7	70.2	11.70	1 30	0.10	18.5	Fore periods without added
8–14	70.0	12.44	1.30	0.06	18.6	arginine.
15–21	70.2	12.15	1.31	0.05	18.7	Periods of arginine adminis-
22–28	70.1	12.32	1.32	0.05	18.8	tration. 1.606 gm. argi-
29–35	69.6	12.29	1.32	0.09	19.0	nine monochloride and 0.64
36–42	70.2	12.98	1.30	0.09	18.5	gm. sodium bicarbonate
43–49	70.1	12.44	1.27	0.11	18.1	administered daily in two
50–56	70.7	11.93	1.34	0.06	19.0	equal doses.
57–63	70 4	12.60	1.33	0.06	18.9	
64–70	70.3	12.77	1.28	0 02	18.2	
71–77	70.6	12.30	1.26	0.01	17.8	After periods without added
78–84	70 9	11.48	1.27	0.02	17.9	arginine.

* The figures represent daily averages for the periods indicated.

7 days duration. The arginine administration was begun after two preliminary periods, and was continued for six and eight periods in the male and female subjects respectively. Two after periods without arginine concluded each experiment. Thus the subjects received amounts of free arginine equivalent to 42 and 56 gm. of creatine respectively. Despite these intakes the data afford no evidence of increased production of creatine or creatinine. With the male subject the creatinine output shows a slight but

distinct decrease which is difficult to explain. During three periods questionable traces of creatine are indicated. Inasmuch as one period of apparent creatine excretion occurred before the arginine administration had begun, and one after it had been discontinued, and since all three appearances were followed immediately by periods during which creatine was entirely absent from the urine, it is extremely unlikely that the ingestion of the amino acid was the responsible factor.

With the female the data are more uniform. In this subject the elimination of creatine and creatinine during the arginine feeding compares quite closely with the values secured during the fore and after periods. It is generally recognized that women are prone to manifest creatinuria. As was pointed out by Rose, Ellis, and Helming, this appears to be due to the fact that the powers of retention and storage of creatine, or the ability to catabolize it by methods which do not yield creatinine, are less effective in women than in men. Women would appear, therefore, to be ideal subjects for the type of experiment under consideration. It was because of this fact that we continued the arginine administration to our female subject 2 weeks longer than to the male. We wished to afford an abundance of time for the manifestation of an exaggerated creatine synthesis if such were possible. Neither the creatine nor creatinine data, however, suggest such a change. Thus the results are in striking contrast to the observations made upon the same female following the administration of corresponding daily doses of creatine (Rose, Ellis, and Helming). As a result of creatine feeding for 7 weeks, 6.0 gm. of extra creatine and 14.6 gm. of extra creatinine were excreted in the urine. It appears, therefore, that if an exaggerated creatine production is induced by arginine feeding, the amount of the former which arises in the human organism is not sufficient to exceed the limited powers of retention of the female. It is possible that other species, particularly the pig, may differ in their response to arginine feeding. On the other hand, the observation of Bunney and Rose (1928) to the effect that this amino acid is probably not essential for the growth of rats, suggests that in the latter the synthesis of tissue creatine does not depend upon the presence of exogenous arginine. In a later communication we shall have more to say concerning the

dispensable nature of arginine and its relation to the creatine-creatinine problem.

SUMMARY.

The daily administration of an amount of arginine equivalent to 1 gm. of creatine exerted no influence upon the output of creatine or creatinine in a male and a female human subject despite the fact that the feeding of the amino acid in the two experiments was continued for 6 and 8 weeks respectively. The results offer no evidence in support of the belief that in man exogenous arginine is catabolized to creatine or creatinine.

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INTERFACIAL ADSORPTION AS A FUNCTION OF THE CONCENTRATION OF COLLOIDAL SOLUTIONS.

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It is the object of this paper to show that the equilibrium values which are obtained by means of the sessile bubble method of measuring surface tension, when a substance like sodium oleate is concentrated at a solution-air interface, are dependent upon the solution concentration. It has heretofore been generally thought that this surface concentration proceeds so as to give surface tension values which are constant, irrespective of the concentration of the solutions, and that the process of adsorption is an irreversible one.

It is a well known fact that certain types of substances, when dissolved, lower the surface tension of water and are absorbed and concentrated at the interface bounding the solutions and air. For compounds of a low molecular weight this process of adsorption is a reversible one so that the material thus concentrated at the interface remains in mobile equilibrium with the bulk of the solution. This condition must not be confused with such cases in which this surface concentration causes denaturation of the substance adsorbed and practically causes the formation of a new phase which is no longer in equilibrium with the bulk of the solution.

By the application of the second law of thermodynamics and the use of data obtained by Whatmough (1) in measuring the surface tension of solutions of acetic acid, Milner (2) has demonstrated that the surface tension of such solutions varies in such a manner that these surface tension values can be expressed as a linear function of the logarithm of the concentration and that the surface excess must therefore be constant at all concentrations.

Milner then used the capillary rise method to note whether the surface concentration of sodium oleate follows a similar procedure. He found, however, that the values which he obtained, down to a concentration of 0.002 N, were such as to "show no trace of any finite value" for the relationship between the change of surface tension with changing solution concentration and the change in solution concentration, that these values "seem to negative the idea of a surface excess," and indicate the "want of reversibility." Similar data have more recently been obtained by the writer (3) by the careful use of the capillary rise method.

There has always been the suggestion that neglect of the angle of contact may well prevent accurate values from being obtained. It is unlikely, however, that this source of error can alone be held responsible for these unusual results. In measuring this type of solution it has always been found that the meniscus sticks to the walls of the capillary after an apparent equilibrium (4) value had been reached. For this reason no significance could be ascribed to such data as equilibrium values, and their real significance was to show how the surface tension changes with time for solutions of various concentrations. The formation of "surface pellicles" and the eventual continuity between this adsorption layer and that on the wall of the capillary may perhaps be held accountable for these irregular results. How poorly data obtained by the capillary rise method fit in with those obtained by other methods is shown by Fig. 1.

More valuable data than these have been obtained by Harkins, Davies, and Clark (5) by the use of the drop weight method. This method is almost entirely unsuited for measuring the surface tension of colloidal solutions because it is not a static method. Where the surface concentration proceeds slowly and for a long time, as it does in the case of very dilute soap solutions, and those of proteins, it is entirely impossible to obtain true equilibrium values by this method (6). This point of view of the writer has been confirmed by Bigelow and Washburn (7).[1] Results obtained

[1] Since completion of this work a paper by Harkins, H. N., and Harkins, W. D., (*J. Clin. Inv.*, **7**, 263 (1929)) severely criticizes the methods now in use for measuring the surface tension of biological fluids. This criticism is valuable because it confirms similar criticisms made by others during the past few years. Unfortunately they propose to replace methods now

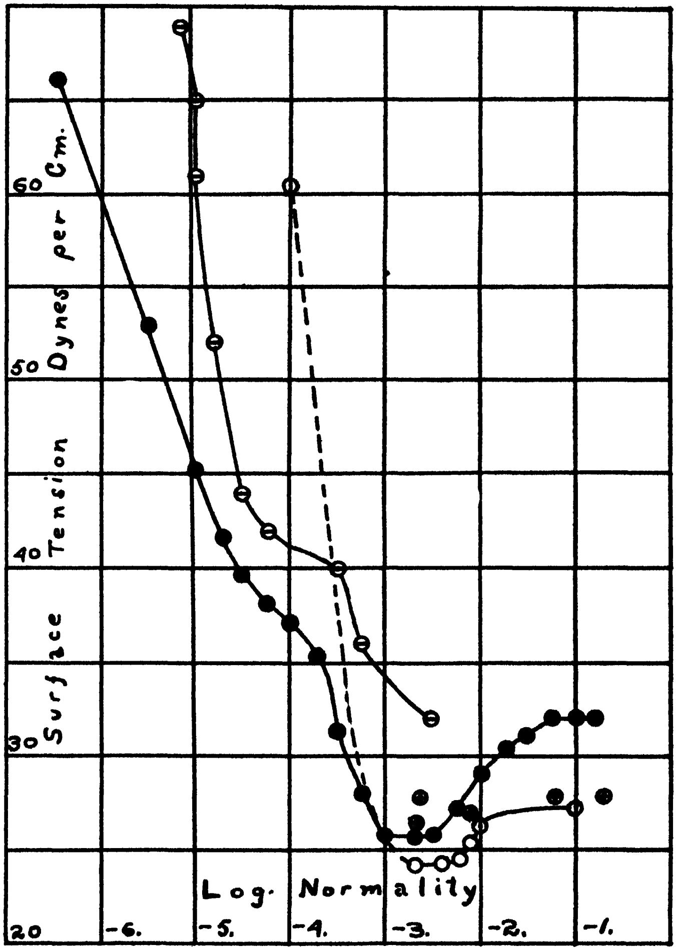

FIG. 1. ●, data with sessile bubble method; ○, drop method of Harkins, Davies, and Clark; ⊖, ring method of du Nouy; ⊕, capillary rise method of Milner.

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in use for obtaining "almost entirely worthless" data by one which, as has been shown in this paper, is equally worthless for obtaining absolute surface tension values of biological solutions. The exceedingly valuable work of W. D. Harkins in other fields of surface tension investigation is so well known that it is to be greatly regretted that he has advocated the use of the drop weight method for this type of solution.

by this method are undoubtedly too high and cannot represent true equilibrium values. In the case of more concentrated soap solutions the surface concentration takes place so rapidly that equilibrium values would be reached very rapidly, so that after a great deal of preliminary cut and try manipulation of the drop weight method, values of relative accuracy might no doubt be obtained. It is most unlikely, however, in the case of dilute solutions where the surface tension is continually changing with time for a long period, first decreasing and usually passing through a minimum and then increasing again, and hours are required for an equilibrium condition to be reached, that even the most careful manipulation can give absolute values which are accurate to the second decimal place. The data which Harkins *et al.* obtained show greater variations, however, than these authors declare and do not warrant their conclusion that "after the concentration of the solution reaches the value 0.002 normal the surface tension no longer decreases but remains constant up to 0.1 normal or more, which proves that the film has become a saturated one." Fig. 1, in which their data are reproduced, shows that this is not the case and how well the variation of their limited amount of data fits in with the larger number of data obtained in the present investigation by means of the sessile bubble method. A lack of better coincidence between these two sets of data can partly be explained by the fact that those obtained by the sessile bubble method are not assumed to represent absolute values, as the sessile bubble method is known to give values which are too high.

The single value which they obtained at the low concentration of 0.0001 N is apparently much too high and involves not only the inaccuracy of the method but also the fact that at this dilution sufficient time was not allowed for the surface concentration to reach its full equilibrium value.

More than 10,000 determinations have been made of the surface tension of solutions of sodium oleate of various concentrations by du Nouy (8), by means of the ring method. Some of the most serious objections to this method have been reviewed before (9). Some of the data taken from du Nouy's curve are reproduced in Fig. 1. It is interesting to note how far his values lie above those of Harkins, Davies, and Clark, even though he allowed a longer time for a condition of equilibrium to be reached than is repre-

sented in their data, which are also too high. Similarly his data are higher than those obtained by the sessile bubble method which also gives values which are known to be relatively high. His values deviate more as his solutions become more dilute, which would indicate that this deviation is due not only to the inaccuracies of the method but also to the want of sufficient time for an equilibrium condition to be reached in the case of these dilute solutions. It should be noted that the general trend of, and the break in, the curve of his data are much like that of the curve representing data obtained by the sessile bubble method.

Present Experiments with the Sessile Bubble Method.—The sessile bubble method was used in the present investigation because it is the only truly static method for measuring surface tension of which the writer is aware. Although the data obtained by this method, given in Fig. 1, do not represent absolute values, they are believed to represent true equilibrium values which show accurately the relative change of surface tension with the change in concentration of solutions of sodium oleate, and this is more than any other method can do. It is to be noted that the surface tension of these sodium oleate solutions becomes constant at concentrations between 0.059 and 0.164 N and that the value obtained at these concentrations does not represent the minimum value for a solution of sodium oleate. Within the range of 0.0000003 to 0.164 N the minimum was found to lie at a concentration of 0.0019 N, which value practically coincides with the concentration at which Harkins and his coworkers found a similar minimum, although they did not consider their data to show sufficient variation to emphasize such a minimum. The number of their determinations was too small to show the exact nature of the change of surface tension with the change in concentration.

The sessile bubble method unfortunately yields data which are known to be much too high. This at least is thought to be the case when this method is used for measuring the surface tension of water and is assumed to be true in the case of other liquids and solutions. No other claim than to values of relative accuracy is therefore made. Even this relative accuracy is none too great, for a considerable change in the surface tension of a solution would be represented by a very small change in the height of the sessile bubble. The application of correction factors in order to obtain

absolute values has not seemed advisable in view of the fact that none of those which have been developed promise to increase the accuracy.

The straight line formed by the data obtained with the most dilute solutions indicates that at these concentrations the surface tension changed with concentration in a similar manner to that in the case of solutions of acetic acid, and that there exists a similar reversibility of the process of adsorption; *i.e.*, the sodium oleate adsorbed at the interface is really in equilibrium with the bulk of the solution. The gradual deviation from a straight line, whatever its caûse, would indicate that even in the case of these more concentrated solutions the material adsorbed at the interface is in equilibrium with the bulk of the solution. The data taken as a whole certainly do not indicate an entire want of reversibility.

Relation between Foam Stability and Surface Tension.—Coincident with the fact that a surface tension minimum occurred at a concentration of 0.0019 N is the fact that this solution formed a foam which was more stable than those of solutions which were either more concentrated or more dilute. A solution at this concentration was, for instance, found to form a foam lasting 3 times as long as one which was 75 times as concentrated. The foams of solutions on either side of this concentration became more stable as their concentration approached it. There was also found a definite point of concentration at which dilute solutions ceased to foam. This point lies at a concentration near 0.000059 N. At this point too, one may observe that there is a brief initial period, following the formation of an interface, during which the surface tension does not change with time. It occurs then, in these dilute solutions, that surface adsorption proceeds so slowly as to make this initial period during which the surface tension of the solution remains that of water, quite readily observed. It is a generally known fact that Lord Rayleigh has shown by rather extraordinary means that such a period of initial inactivity occurs in the case of all solutions which are surface-active. In the case of these dilute soap solutions this initial period can be observed readily by means of the capillary rise method. In the case of the most dilute ones it is possible to observe it when using the sessile bubble method.

Preliminary Experiments with Gelatin.—Data repeatedly obtained with gelatin solutions show the surface tension to vary linearly with the logarithm of the concentration at concentrations of 1 per cent and less. The method has so far not been applied to measuring the relative surface tension of blood sera representative of various conditions.

Apparatus.—The apparatus used in obtaining data by the sessile bubble method is illustrated by Fig. 2. The chief part of the apparatus consisted of a glass cell, *A*, having the dimensions 5 × 5 × 1 cm. The top and bottom were of square pieces of plate

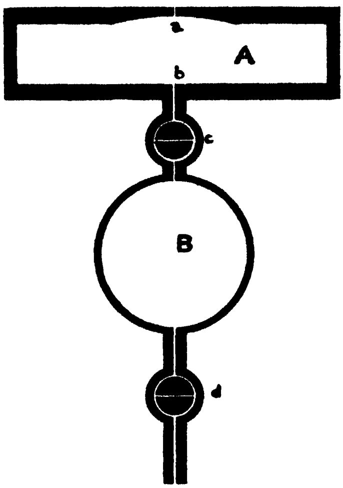

FIG. 2. Apparatus for measuring surface tension of blood serum. *A*, glass cell; *B*, bulb filled with mercury.

glass and the four sides of optically plane glass. All were luted together with acid-alkali-proof cement. A concave depression, like that of a hanging drop slide, in the center of the top, *a*, kept the bubble from sliding off to one side before the apparatus had been properly leveled. A small air hole in the center of this depression permitted the admission of air for the formation of the sessile bubble when a part of the liquid in the cell was withdrawn at the opening, *b*, in the center of the bottom. A glass bulb with stop-cocks, *c* and *d*, on either side of it was attached to the cell at the opening, *b*, by means of a ground joint. The apparatus was mounted on a firm iron support

which could be adjusted so as to insure the proper leveling of the cell.

The cell was cleaned with hot chromic acid before each experiment. It was filled with the solutions to be measured by placing a cover-slip over the air hole at *a;* the solution was introduced through the opening at *b* by means of a capillary pipette. The bulb, *B*, which was attached to a mercury reservoir, was completely filled with clean mercury. The cell with its charge was then mounted at the ground joint and sufficient solution allowed to displace the mercury in the bulb below to produce a sessile bubble which would have a diameter of 3.5 to 4.2 cm. when a condition of equilibrium had been reached. The production of a Torricellian vacuum in the bulb, *B*, made it possible to form the sessile bubble with great rapidity.

The point of maximum depth of this bubble was determined by placing a strong light and an intervening screen back of it and reading this point with a traveling microscope which can be used as a cathetometer. The point of maximum diameter of the bubble was read by placing an intense point of light 15 feet distant in exactly the same plane as that of the bubble and at 90° to the line of vision of the microscope.

The actual distance representing the surface tension that is measured is about 15 times greater in the case of the capillary rise method than it is in the case of the present method, and the degree of accuracy of the latter method is therefore correspondingly less. The change in the distance to be measured as the surface tension changes with time is, in the case of the sessile bubble method, so small for a fractional period of the time required for equilibrium that no attempt was made to note how the change with time takes place. It was soon learned that concentrated solutions of sodium oleate came to equilibrium with the interface within an hour. In the case of dilute solutions where hours were required for an equilibrium condition, the experiment was continued until there was no further change in the course of 2 or 3 hours. Successive readings taken at equilibrium could usually be reproduced within 0.01 mm., even when the bubble was shifted about slightly between readings.

For the calculation of the surface tension the simple equation $s = \frac{h^2dg}{2}$ was used. h is the vertical distance from the point of

maximum depth of the bubble to the point of its maximum diameter, s the surface tension measured in dynes per cm., d the density of the solution, and g the acceleration of gravity.

SUMMARY.

The sessile bubble method has been applied for measuring the surface tension of solutions of sodium oleate varying in concentration from 0.164 to 0.0000003 N. For the most dilute solutions, the surface tension varies linearly as a function of the logarithm of the concentration as it does in the case of solutes like acetic acid. As the solutions become more concentrated there is a gradual deviation from this relationship until a surface tension minimum is reached at a concentration of 0.0019 N when surface tension values rise again with increasing concentration until a concentration of 0.059 N is reached. At still higher concentrations the surface tension remains constant up to a concentration of 0.164 N. The values obtained by this method are not absolute. As relative values they nevertheless indicate that there is not an entire want of reversibility between the sodium oleate adsorbed at the interface and the main bulk of the solution. They also show that previous conclusions, that after the concentration of sodium oleate solutions reaches a concentration of 0.002 N the surface tension remains constant, are incorrect. It has also been shown that the stability of foams, produced with sodium oleate solutions, is at a maximum when the surface tension is at a minimum, and that foam stability is a function of surface tension rather than of solution concentration. It has been demonstrated again that there is a sharply defined limiting concentration below which soap solutions do not form foams.

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THE FORMATION OF GLYCOGEN IN THE LIVER OF THE YOUNG WHITE RAT AFTER THE ORAL ADMINISTRATION OF GLYCEROL.

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Several substances possessing 3 carbon atoms are generally thought to play a part in the intermediary metabolism of carbohydrates. The rôle of glycerol has been investigated by several authors. Since the work of Cremer (1) and more recently of Chambers and Deuel (2) on phlorhizinized animals, and that of Luthje (3) on depancreatized dogs, it has been generally agreed that ingested glycerol is excreted as glucose in the urine by the diabetic organism. Deposition of liver glycogen after administration of glycerol, however, has not been satisfactorily demonstrated. Pfluger (4) in 1905 reviewed critically the work of earlier investigators who had reported the formation of significant amounts of glycogen after the administration of glycerol and concluded that glycogen formation in the liver after administration of glycerol was insignificant. On perfusion of the liver of a phlorhizinized dog with a 20 per cent glycerol solution, Barrenscheen (5) noted a slight loss in liver glycogen and an insignificant rise only in the sugar content of the perfusion fluid. Doyon and Morel (6) found no change in the glycogen content of dog liver after injection of glycerol into an intestinal vein over a period of 50 minutes.

Because this evidence concerning the rôle of glycerol in carbohydrate metabolism is inconsistent, and in view of the suggestive similarity in structure between glycerol and such known products of glucose catabolism as glyceric aldehyde and dihydroxyacetone, a further inquiry into the possibility of glycogen formation from glycerol was thought advisable. In the work presented here the

problem of glycerol utilization has been approached by a study of the glycogen formation in the livers of young white rats after the oral administration of glycerol We obtained definite and prompt deposition of liver glycogen, increasing in amount up to 6 hours after feeding. The results indicate that under our experimental conditions glycerol is readily utilized in the formation of liver glycogen.

EXPERIMENTAL.

The experimental procedure was similar to that employed by Cori (7) in his studies of the absorption and metabolism of hexoses. Young white rats, weighing for the most part between 100 and 150 gm., were placed upon a standard diet of leaf lettuce, whole wheat bread, and milk. Prior to the experimental feeding period, the rats were placed in individual cages and fasted for 24 hours, an adequate supply of water being kept available at all times. The control rats were fed approximately 2 cc. of water by stomach tube, with the exception of Rats 4, 5, and 6 which were not handled. The experimental animals received 2 cc. of a 50 per cent glycerol solution by stomach tube, an amount of glycerol which caused no symptoms of gastrointestinal disturbance. With this amount, some glycerol was always observed to remain in the stomach at the end of the experimental period. In order to obtain evidence concerning the relative rates of glycogen formation from glycerol and glucose, nine rats were fed 2.5 cc. of a 50 per cent glucose solution. All of the animals were killed with chloroform, the control rats shortly after the administration of water, the others after periods of absorption as indicated in Tables II and III. The livers were quickly removed and their glycogen content determined by Pflüger's method. Glucose resulting from the hydrolysis of the glycogen was determined by the method of Hagedorn and Jensen (8).

DISCUSSION.

Our results are summarized in Tables I to III. Most of the rats used were males, but since sex did not appear to influence the results, this factor was disregarded in the tables. The livers of the rats receiving water only showed an average glycogen content of 0.09 per cent, an amount equivalent to 3.40 mg. per

100 gm. of body weight. When glycerol was fed, a significant increase in liver glycogen was noted (Table II), the average percentage rising progressively from 0.29 after 1 hour's absorption to 3.24 after 6 hour absorption periods; expressed as mg. per 100 gm. of body weight, glycogen values rose from 12.93 to 142.55. The increase of liver glycogen to 3 times that of the controls within an hour and the progressively higher figures for longer periods of absorption indicate a definite and prompt utilization of ingested glycerol.

TABLE I.

Glycogen Content of Livers of Control White Rats after Fasting Periods of 24 Hours.

Rat No.	Weight after fast.	Loss in weight.	Weight of liver.	Glycogen of liver.		
	gm	*gm.*	*gm.*	*mg.*	*per cent*	*mg. per 100 gm. body weight*
1	131	17	6 12	3.64	0.06	2.77
2	135	14	5.88	3.09	0.05	2 29
3	178	17	6 81	3.24	0.05	1.82
4	98	11	3.66	5 69	0.15	5.80
5	164	14	5.99	14.09	0.23	8.59
6	101	12	3 74	5 16	0.14	5.11
7	169	9	5 30	3.78	0 07	2.24
8	116	10	4.56	2.59	0.05	2.23
9	167	20	6.93	8.00	0.11	4.79
14	169	10	5 92	3.90	0.06	2.31
17	185	15	5.54	2.73	0.05	1.48
20	130	12	4.71	3.15	0.07	2.42
24	109	15	4.52	3.24	0.07	2.97
32	111	19	5 04	3 61	0.07	3.25
40	115	13	4.41	3.75	0.08	3.26
44	108	11	4 23	3 35	0.08	3.10
Average....				4.56	0 09	3.40

It may be seen that with a single exception (Rat 9), less than 4 mg. of glycogen was found in the livers of control rats receiving the same treatment as experimental animals (*i.e.* administration of 2 cc. of water in place of the glycerol), and that somewhat higher values were obtained for Rats 4, 5, and 6, which received no water. The results obtained for the glycogen content of the

TABLE II.

Glycogen Content of Livers of White Rats after Oral Administration of Glycerol.

Rat No.	Weight after fast.	Loss in weight.	Period of absorption.	Weight of liver.	Glycogen of liver.		
	gm.	*gm.*	*hrs.*	*gm.*	*mg.*	*per cent*	*mg. per 100 gm. body weight*
10	127	8	1	5.01	20.55	0.41	16.18
11	131	11	1	4.72	3.43	0.07	2.62
23	124	18	1	6.06	19.35	0.32	15.60
26	136	12	1	6.20	3.75	0.06	2.76
37	113	19	1	5.65	26.88	0.47	23.78
41	105	12	1	4.31	17.45	0.40	16.62
Average					15.23	0.29	12.93
15	140	19	2	6.23	44.22	0.71	31.58
25	114	12	2	4.59	44.96	0.98	39.44
27	131	13	2	5.71	97.33	1.70	74.29
29	111	16	2	5.19	94.55	1.82	85.18
42	125	14	2	4.85	55.80	1.15	44.64
46	111	13	2	5.12	45.21	0.88	40.73
Average					63.68	1.21	52.64
12	124	12	3	5.08	60.21	1.18	48.56
13	137	14	3	5.87	82.97	1.41	60.56
16	127	14	3	5.34	92.70	1.74	72.99
18	112	14	3	4.55	113.79	2.50	101.59
22	117	13	3	5.48	120.51	2.19	103.00
33	107	16	3	5.44	122.83	2.26	114.79
Average					98.83	1.88	83.58
28	106	10	4	4.56	146.23	3.21	137.95
31	107	10	4	4.96	37.05	0.75	34.62
34	108	13	4	4.46	25.77	0.58	23.86
36	109	16	4	5.30	190.96	3.60	175.19
39	117	15	4	5.23	174.28	3.33	148.96
43	99	12	4	4.83	136.73	2.83	138.11
Average					118.50	2.38	109.78
19	167	17	6	5.50	90.61	1.65	54.25
21	140	12	6	5.45	109.38	2.00	78.13
30	130	16	6	6.04	316.11	5.23	243.16
35	108	16	6	5.06	190.96	3.77	176.81
38	110	12	6	4.49	107.53	2.39	97.75
45	117	14	6	5.42	240.09	4.43	205.20
Average					175.78	3.24	142.55

livers of the control rats after 24 hours of fasting in our series are somewhat lower than those obtained by other investigators. Although it is not definitely stated, it is probable that the control animals in these studies (9, 10) were not handled as was the case in our own experiments in connection with the use of the stomach tube.[1] The feeding apparently caused little discomfort, but it is possible that a part of the liver glycogen was mobilized or deposition slightly decreased as a result of handling the rats.

The rather wide differences in the amounts of glycogen deposited in the livers of rats during comparable absorption periods

TABLE III.

Glycogen Content of Livers of White Rats after Oral Administration of Glucose.

Rat No.	Weight after fast.	Loss in weight.	Period of absorption.	Weight of liver.	Glycogen of liver.		
	gm.	*gm.*	*hrs.*	*gm.*	*mg.*	*per cent*	*mg. per 100 gm. body weight*
48	99	14	2	4.13	66.28	1.60	66.95
50	113	16	2	4.70	60.72	1.29	53.73
52	98	13	2	3 88	46.35	1.19	47.29
54	99	14	2	4.55	45.42	0.99	45.88
Average					54.69	1.27	53.46
47	104	12	3	4.39	100.12	2.28	96.27
49	109	9	3	4.55	80.42	1.77	73.78
51	102	13	3	4.02	69.52	1.73	68.16
53	93	12	3	3.88	82.27	2.12	88.46
55	109	12	3	4 50	80.42	1.78	73.78
Average					82.55	1.94	80.09

are to be expected, since neither rate of absorption nor metabolism by other routes, such as oxidation or glycogen formation in the rest of the body, was followed. Rats 11 and 26 of the 1 hour absorption period series showed amounts of liver glycogen similar

[1] The possibility suggested itself that the low glycogen values observed might be due to increased glycogenolysis incidental to use of chloroform in killing the rats. However, the results of the experiments of Wilson (11), in which the rats were killed by a blow on the head or by chloroform, do not indicate that the use of chloroform caused appreciable changes in the glycogen content of the liver.

to those of the controls; here it seems probable that the glycerol was poorly absorbed.

The rats fed glucose, as shown in Table III, deposited amounts of liver glycogen quite comparable to those formed by the animals absorbing glycerol over similar periods. Thus the 2 hour groups showed an average of 1.27 per cent of liver glycogen after glucose feeding as compared with 1.21 per cent when glycerol was fed, and the rats killed 3 hours after feeding had average values of 1.94 per cent after glucose ingestion and 1.88 per cent after glycerol. Glycogen values in mg. per 100 gm. of body weight are also similar. It would seem that under the conditions of our experiments, for the 2 and 3 hour absorption periods at least, glucose and glycerol are almost equally good sources of liver glycogen.

Noble and Macleod (12) have reported that 10 cc. of glycerol injected into a rabbit as an antidote for insulin convulsions did not increase blood sugar or favorably influence the convulsions. Voegtlin, Dunn, and Thompson (13, 14), on the other hand, demonstrated both a curative and a preventive action of glycerol in insulin hypoglycemia when the alcohol was administered to white rats in 20 per cent solution either intraperitoneally or by slow intravenous injection. The same authors (15) have noted a hyperglycemia in rabbits after administration of glycerol both orally and intraperitoneally, and have also shown in insulinized rabbits a beneficial action of glycerol when injected intraperitoneally (13) or fed (15) in 20 per cent solution. They recognize that to achieve any curative action in insulin coma or convulsions the therapeutic agent must be promptly available, and state that if glycerol is transformed to glucose, the conversion must be very rapid. In view of the similarity in the rate of deposition of liver glycogen after the administration of glucose and glycerol in our own studies, we believe that our results tend to support the view that glycerol may be an efficient antidote for insulin intoxication.

SUMMARY.

Young white rats after fasting periods of 24 hours were fed glycerol by stomach tube. The content of glycogen in the livers at periods from 1 to 6 hours after the ingestion of glycerol increased progressively to an average maximum value of 3.24 per cent, or 142.55 mg. per 100 gm. of body weight. The livers of

control rats, which received water only, contained an average of 0.09 per cent of glycogen. The amounts of liver glycogen after 2 and 3 hour periods of absorption of glycerol were similar to those observed after absorption of glucose over the same periods. Under our experimental conditions glycerol appears to be readily metabolized and deposited as glycogen in the livers of young white rats.

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METABOLISM OF AMINES.

I. TRIMETHYLAMINE.

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The simple aliphatic amines are known generally to be toxic substances and to have little therapeutic use. They are formed during bacterial decomposition of most animal or vegetable matter, and may be detected easily because of their volatility and characteristic odors. The amine most frequently met with in decomposed organic matter is trimethylamine. It is liberated also upon destructive distillation of certain tissues. Trimethylamine has been thought to be present in small amounts in the blood and urine of mammals (1). Takeda (2) and Erdmann (3) have shown by working with fresh blood and urine that this is not the case. Trimethylamine oxide has been isolated several times from fish muscle and urine (4–9). Suwa (10) has injected trimethylamine oxide into rabbits, and has isolated both trimethylamine and the unchanged oxide from the urine. This finding would lead one to suspect that trimethylamine is not readily metabolized by mammals, but is excreted unchanged. Neither the free amine nor the amine oxide has been found in fresh mammalian tissue. The occurrence of trimethylamine combined in the form of quaternary ammonium bases among the extractives of various tissues is well known. Such bases are betaine, carnitine, choline, ergothioneine, neosine, and trimethylaminocrotonic acid.

The principal chemical decomposition which characterizes a quaternary ammonium base is the liberation of a tertiary amine on heating with alkali. Kohlrausch (11) has shown that betaine, when fed to rabbits, gives rise to trimethylamine, and it is conceivable that each of the quaternary ammonium bases named above may decompose similarly in the body so as to liberate

trimethylamine. It is not improbable that the physiological functions of the substances may be associated with such a chemical transformation in which trimethylamine is liberated. In view of these considerations, it is surprising that quantitative studies of the metabolism of trimethylamine itself have not been made.

A review of the analytical procedures for the separation and determination of each of the methylamines and of ammonia in a mixture has been made by Weber and Wilson (12). These workers proved that the procedures described in the literature prior to 1918 led to inaccurate results, and they developed a new quantitative method for the determination of each component in a mixture of the four bases. Woodward and Alsberg (13) have since developed a method for the determination of trimethylamine only in the presence of the other amines and ammonia, but the method offers no improvement over that of Weber and Wilson.

The present work is an application of the procedure of Weber and Wilson to a study of the metabolism of trimethylamine by rabbits. The rabbits were maintained upon a diet of carrots, and the nitrogen intake was reduced to less than 1 gm. per day. Weighed amounts of the amine hydrochloride dissolved in small amounts of water were given through a stomach tube, and sodium carbonate in amounts sufficient to prevent the depletion of base in the tissues was given immediately after. The urine of the rabbits was then collected in 24 hour periods and was analyzed for ammonia, the amines, and its total nitrogen content. In order to avoid loss of the volatile amines from the urine, and also to prevent bacterial decomposition, benzoic acid was placed in the bottles used for collecting the urine.

After the measurement of the volume of the urine and the removal of samples which were used for the determination of total nitrogen, magnesium oxide was added, and the alkaline urine was distilled *in vacuo*. Octyl alcohol was used to prevent frothing. The distillate was collected in standard acid contained in a receiver which was cooled with ice. When about 200 cc. of distillate were obtained, the excess of standard acid was titrated with sodium hydroxide. The acid which was neutralized during the distillation was a measure of the total volatile base. The

solution of bases then was analyzed for ammonia and the amines as follows:[1]

The solution was made alkaline with a mixture of sodium hydroxide and sodium carbonate, and was diluted to a known volume. Yellow mercuric oxide was added so as to give a fine suspension in the alkaline solution. The flask was covered to exclude light and was shaken for 1 hour; the suspension then was allowed to settle for several hours. The solution was filtered through cotton by use of air pressure, and aliquot portions of the filtrate were distilled into standard acid. The acid neutralized during the distillation was a measure of the total amines. The difference between the total volatile base and the total amines was considered to be ammonia.

The aliquot distillates containing the volatile amines were combined, and the solution was acidified with sulfuric acid and evaporated on a steam bath to about 25 to 30 cc. It was treated with an excess of sodium nitrite and glacial acetic acid. The nitrous acid produced decomposed the primary amine, converted the secondary amine into the nitrosoamine, and left the tertiary amine unchanged. The solution containing the nitrous acid was allowed to stand for about 12 hours, and then was made alkaline with sodium hydroxide and distilled into standard acid. The acid neutralized was a measure of the tertiary amine present. Any nitrosoamine which was formed from secondary amine was now to be found mixed with the tertiary amine in the distillate. The nitrosoamine, however, was indifferent toward the acid and alkali used in the titration, so that the values for the tertiary amine were not affected by its presence.

To the solution containing nitrosoamine and tertiary amine after the titration were added concentrated hydrochloric acid and metallic zinc, in order to reduce the nitrosoamine to the secondary amine again. The solution was allowed to stand for about 24 hours while the evolution of hydrogen continued. It then was made alkaline by the addition of sodium hydroxide, and the amines were distilled again into standard acid. The acid neutralized was a measure of both the secondary and tertiary amines. The difference between the titration values before and after re-

[1] For the preparation of solutions and for other details consult the paper by Weber and Wilson.

duction represented secondary amine. The primary amine was assumed to be the difference between the total amines and the sum of secondary and tertiary amines. This method of estimating the primary amine was different from the procedure of Weber and Wilson, and it made the analyses less accurate than theirs. However, by use of this modification, the analyses could be made in much less time, and by a more simple process.

It was found that a good commercial grade of yellow mercuric oxide failed to absorb ammonia from solution. Fresh oxide was therefore prepared from mercuric chloride by addition, while cold, of slight excess of sodium hydroxide. Ice was used in the reaction mixture to keep the temperature down. The precipitate which formed was brown, and changed to yellow when the last portions of alkali were added. The yellow oxide thus obtained was filtered with suction, washed thoroughly with water, and kept moist. It absorbed ammonia rapidly.

The absorption of ammonia by the mercuric oxide, while rapid, was not quantitative. There usually remained in the alkaline solutions sufficient unabsorbed ammonia to give values for the daily output of a rabbit, 1 to 2 mg. of ammonia nitrogen too low. The failure to absorb ammonia quantitatively was shown not to be due to the presence of octyl alcohol used to prevent frothing during the distillation of the urine. The error in the determination made unreliable the values for the primary amine.

Determinations of urea were made by the urease-aeration method. The benzoic acid present in the urine was shown to have no apparent inhibitory action upon the urease.

The analytical data are presented in Tables I to IV. Table I shows the effect of the diet alone upon the excretion of nitrogen in the urine. The nitrogen content of the carrots was calculated from the data of Atwater and Bryant (14), who determined that carrots contain 1.1 per cent protein. The calculation was made on the assumption that this protein of carrots was 16 per cent nitrogen. Tables II and III show the recovery of the amines after ingestion of trimethylamine, and Table IV the variation in urea and ammonia plus trimethylamine after feeding trimethylamine. Table II was taken as a short period from a much more prolonged experiment. The rabbits had been maintained upon carrots *ad libitum* for 30 days before the start of the period pre-

TABLE I.

Excretion of Nitrogen in Urine of Rabbits Maintained upon a Diet of Carrots.

Date.	Volume.	N intake.	N output.	Urine volume.	N intake.	N output.
	Rabbit 1, female.			Rabbit 2, male.		
1929	cc.	gm.	gm.	cc.	gm.	gm.
Feb. 11	580	0.528	0.898	140	0.528	0.835
" 12	680	0.528	0.855	180	0.233	0.673
" 13	630	0.528	0.822	50	0.220	0.935
" 14	600	0.528	0.680	57	0.201	1.065
" 15	768	0.528	0.738	48	0.334	0.813
" 16	695	0.528	0.680	188	0.448	0.657
" 17	580	0.528	0.736	425	0.492	0.630
" 18	465	0.528	0.817	397	0.528	0.578
" 19	640	0.528	0.866	443	0.528	0.623
" 20	570	0.528	0.634	350	0.528	0.469
" 21	345	0.528	0.706	320	0.528	0.697
" 22	325	0.601	0.721*	267	0.601	0.713*
" 23	310	0.601	0.674*	325	0.601	0.648*
" 24	620	0.528	0.651	410	0.528	0.581
" 25	595	0.528	0.654	325	0.528	0.560
	Rabbit 3, male.			Rabbit 4, male.		
Feb. 11	378	0.528	0.656	228	0.704	0.858
" 12	353	0.528	0.651	440	0.704	1.108
" 13	253	0.528	0.688	310	0.704	0.871
" 14	393	0.528	0.916	225	0.704	0.476
" 15	295	0.528	0.753	485	0.704	0.819
" 16	575	0.528	0.533	405	0.704	0.688
" 17	360	0.528	0.624	252	0.704	0.496
" 18	240	0.528	0.695	388	0.704	0.780
" 19	240	0.528	0.672	407	0.704	0.998
" 20	258	0.528	0.906	390	0.704	0.682
" 21	295	0.528	0.820	522	0.704	1.314
" 22	222	0.601	0.752*	480	0.676	1.326†‡
" 23	234	0.601	0.792*	370	0.792	1.237†
" 24	400	0.528	0.770	595	0.704	1.147
" 25	247	0.528	0.705	745	0.704	1.350

Rabbit No.	1	2	3	4
	kg.	kg.	kg.	kg.
Feb. 11	2880	2800	2545	3785
" 25	2600	2470	2280	3325

The carrot diet was started February 7.

* 0.50 gm. of $(CH_3)_3N \cdot HCl$ and 0.20 gm. of Na_2CO_3 were given.

† 0.60 gm. of $(CH_3)_3N \cdot HCl$ and 0.25 gm. of Na_2CO_3 were given.

‡ N from carrots = 0.588 gm.

sented. Trimethylamine had been given at intervals, and the urine had been analyzed throughout the 30 days. The figures presented in Table II are typical of the figures for the entire period. Table III was constructed from data obtained upon two rabbits which had not been experimented upon previously. Rabbit 3 was young and growing; Rabbit 4 was large and mature.

TABLE II.

Recovery of Amines from Urine of Rabbits after Ingestion of Trimethylamine.

Date.	Volume.	N intake.	N output.	Base.	Amines.	NH_3	R_2NH	$(CH_3)_2NH$	R_3N	$(CH_3)_3N$	$(CH_3)_3N$ recovery.
					Rabbit 1, female.						
1928	*cc.*	*gm.*	*gm.*	*cc. N acid*	*cc. N acid*	*gm.*	*cc. N acid*	*gm.*	*cc. N acid*	*gm.*	*per cent*
Nov. 26	270	0.528	0.427	0.048					0.031		
" 27	270	0.587	0.675	0.449	0.353	0.001	0.027	0.001	0.297	0.017	6.8*
" 28	440	0.587	0.528	0.953	0.658	0.005	0.041	0.002	0.276	0.016	6.4*
" 29	495	0.587	0.550	0.563	0.488	0.001	0.036	0.002	0.429	0.025	10.8*
" 30	540	0.528	0.417	0.151	0.136	0.000			0.011		
Dec. 1	495	0.528	0.407	0.071	0.080				0.021		
					Rabbit 2, male.						
Nov. 26	270	0.528	0.552	0.183	0.092	0.001					
" 27	185	0.587	0.508	1.539	1.528	0.000	0.015	0.001	1.190	0.070	2.8*
" 28	230	0.587	0.631	1.682	1.248	0.007			0.949	0.055	2.2*
" 29	260	0.587	0.624	0.816?	1.223		−0.005	0.000	0.777	0.046	1.8*
" 30	230	0.528	0.508	0.993	0.183	0.013					
Dec. 1	193	0.528	0.465	0.088	0.048				0.027		

* 0.400 gm. of $(CH_3)_3N \cdot HCl$ and 0.15 gm. of Na_2CO_3 were given.

The data (Table I) show that mature rabbits which were maintained upon a constant intake of carrots did not come to nitrogen equilibrium, at least by the end of 11 days, but remained in negative nitrogen balance. When the nitrogen intake was kept constant, the nitrogen excretion varied greatly, so that changes resulting from the ingestion of amine could not be detected with certainty. There seemed to be some evidence that the ingestion of trimethylamine by rabbits reduced the negative nitrogen bal-

TABLE III.

Recovery of Amines from Urine of Rabbits after Ingestion of Trimethylamine.

Date.	Volume.	N intake.	N output.	Base.	Amines.	NH_3	R_2NH	$(CH_3)_2NH$	R_3N	$(CH_3)_3N$	$(CH_3)_3N$ recovery.
Rabbit 3, male.											
1928	*cc.*	*gm.*	*gm.*	*cc. N acid*	*cc. N acid*	*gm.*	*cc. N acid*	*gm.*	*cc. N acid*	*gm.*	*per cent*
Dec. 20	133	0.528	0.428						0 015	0.000	
" 21	170	0.528	0.432	1.062	0.044	0.017					
" 22	170	0.528	0.425	0.283	0.055	0.004	0.024	0.001	0.016	0.009	
" 23	250	0.572	0.511	0.698	0.435	0.004	0.041	0.002	0.186	0.109	5 9*
" 24	175	0.572	0.496	1.641	0.791	0.014	0.130	0.006	0.132	0.008	4.4*
" 25	220	0.572	0.493	0.281	0.198	0.001			0.156	0 009	4 9*
" 26	246	0.572	0.480	0.749	0.630	0.001	0.054	0.002	0.452	0.026	14 2*
" 27	240	0.572	0.437	0.496	0.344	0.002	0.047	0.002	0.264	0.015	8 2*
" 28	225	0.601	0.480	1.521	1.125	0.007	0.003	0.000	1.029	0.060	19 3†
" 29	205	0.601	0.494	1.271	0.746	0.009	0.088	0.004	0.596	0.035	11.3†
" 30	218	0.528	0.338	0.589	0.191	0.006	0.029	0.000	0.006	0.000	
" 31	176	0.528	0.328	0.362	0.066	0.005	0.006	0.000	0.036	0.002	
Rabbit 4, male.											
Dec. 20	360	0.704	0.994	0.135	0.112	0.000			0.011	0.000	
" 21	300	0.704	0.865	0.112	0.055	0.001	0.000		0.019	0.001	
" 22	270	0.704	0.817	0.200	0.156	0.000			0.024	0.001	
" 23	350	0.792	1.064	0.402	0.360	0.001	0.080	0.003	0.250	0.014	3.7‡
" 24	288	0.792	1.010	0.616	0.545	0.001	0.110	0.005	0.229	0.013	3.6‡
" 25	260	0.792	0.858	1.558	1.045?	0.008			0.171	0.010	2.7‡
" 26	400	0.792	1.172	1.256	1.031	0.004			0.566	0.033	9.0‡
" 27	340	0.792	0.941	1.493	0.350	0.019	−0.005	0.000	0.514	0.030	8.1‡
" 28	312	0.792	0.889	2.042	0.475	0.026	0.051	0.002	0.398	0.023	6.8‡
" 29	313	0.792	0.825	1.401	1.028	0.006	0.002	0.000	0.894	0.052	14.2‡
" 30	174	0.704	0.326	0.522	0.043	0.008			0.042	0.002	
" 31	330	0.704	0.631	0.250	0.048	0.003					

* 0.300 gm. of $(CH_3)_3N \cdot HCl$ and 0.120 gm. of Na_2CO_3 were given.
† 0.500 gm. of $(CH_3)_3N \cdot HCl$ and 0.200 gm. of Na_2CO_3 were given.
‡ 0.600 gm. of $(CH_3)_3N \cdot HCl$ and 0.250 gm. of Na_2CO_3 were given.

ance, so that one might conclude that the trimethylamine had a nitrogen-sparing action. However, the opposite conclusion, that trimethylamine had a stimulating effect, resulting in an output of nitrogen above that expected, might be drawn from the data of Table III.

From 80 to 96 per cent of the trimethylamine ingested was changed to other substances, 4 to 20 per cent being excreted un-

TABLE IV.

Excretion of Urea and of Ammonia Plus Trimethylamine after Ingestion of Trimethylamine.

Date.	Volume.	N intake.	N output.	Urea N.	$NH_3 + (CH_3)_3N$ N	Extra urea N.
			Rabbit 1, female.			
1929	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
June 15	458	0.528	0.765	0.542	0.008	
" 16	467	0.601	1.006	0.695	0.008	0.080*
" 17	560	0.528	0.815	0.441	0.009	
" 18	423	0.675	1.077	0.733	0.047	0.145†
" 19	570	0.528	0.680	0.362		
			Rabbit 2, male.			
June 15	233	0.501	0.604	0.456	0.004	
" 16	215	0.565	0.692	0.490	0.006	−0.033*
" 17	295	0.511	0.604	0.459	0.003	
" 18	280	0.675	0.751	0.470	0.053	−0.077†
" 19	385	0.528	0.632	0.394		
			Rabbit 3, male.			
June 15	414	0.528	0.646	0.499	0.011	
" 16	320	0.601	0.739	0.495	0.009	−0.018*
" 17	417	0.528	0.720	0.550	0.014	
" 18	313	0.675	0.918	0.558	0.065	−0.074†
" 19	420	0.528	0.862	0.645	0.010	

By "extra urea N" is meant the amount above that estimated to have been formed from the N of the amine metabolized.

The rabbits had been maintained on a constant intake of carrots since June 10.

* 0.500 gm. of $(CH_3)_3N \cdot HCl$ and 0.20 gm. of Na_2CO_3 were given.

† 1.00 gm. of $(CH_3)_3N \cdot HCl$ and 0.40 gm. of Na_2CO_3 were given.

changed. The metabolism of this amine, as measured by the excretion of its nitrogen, was practically complete within 24 hours. Even when the amine was ingested for several successive days, it

did not "accumulate," so that the excretion for each day could be said to contain the products from the amine ingested on that day.

The amount of dimethylamine excreted after the ingestion of trimethylamine was almost insignificant. The dimethylamine recovered during all experiments representing the amines excreted after the ingestion of over 20 gm. of trimethylamine hydrochloride, was separated from the trimethylamine by distillation of the nitrosoamine from acid solution. The nitrosoamine was then reduced and determined as usual. The amount of secondary amine thus formed corresponded to only 0.216 cc. of N solution, which was equivalent to but 9 mg. of dimethylamine. An attempt to prepare the *p*-bromobenzenesulfonamide of the amine in this distillate was not successful.

The amount of monomethylamine recovered after ingestion of trimethylamine is less than might be found merely from the experimental error of the method. It is to be expected that if the primary amine were formed, it would be deaminized rapidly. Separate determinations which are not reported in this paper indicate that no monomethylamine could have been excreted during these experiments.

The ammonia excreted after the ingestion of trimethylamine was generally not more than was excreted when the amine was not ingested. For reasons which are not known, the amount of ammonia excreted varied greatly. Bacterial decomposition of the urine may have occurred or some ammonia may have been formed from urea during the distillation of the urine. In order to determine the effect of the metabolism of the amine upon the excretion of ammonia, other experiments must be performed.

The trimethylamine that is metabolized by rabbits gives rise to an increased output of urea (Table IV). The increase of urea nitrogen may be greater or less than the nitrogen of the amine metabolized. No explanation of this variation is offered at present. It may be that the urea output is independent of the metabolism of the amine.

SUMMARY.

Trimethylamine, when fed to rabbits in amounts up to 125 mg. of free base per kilo of rabbit, is metabolized to the extent of 80 to

96 per cent of the amine fed, the remainder being excreted as the unchanged amine.

Dimethylamine is excreted in only minute amount after the ingestion of trimethylamine, while monomethylamine seems not to be excreted at all.

The excretion of ammonia seems to be independent of the amount of trimethylamine metabolized.

Part, if not all, of the nitrogen of trimethylamine which has been metabolized, seems to be excreted as urea.

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ON WALDEN INVERSION.

XIII. THE INFLUENCE OF SUBSTITUTING GROUPS ON OPTICAL ROTATION IN THE SERIES OF DISUBSTITUTED ACETIC ACIDS.

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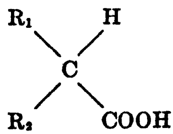
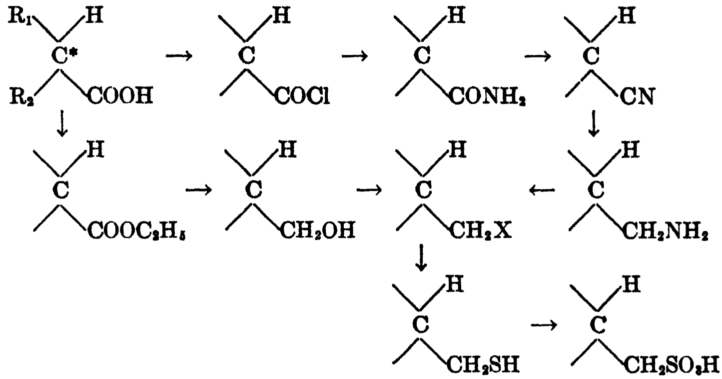
The present investigation was undertaken with the aim of testing the conclusions reached by Levene and Mikeska[1] regarding the configurations of secondary aliphatic carbinols and of halides derived from them by the substitution of the hydroxyl by a halogen. In a previous work the configurations of the carbinols and halides were correlated on the basis of a comparison of the rotations of these substances between themselves and of a parallel comparison of the rotations of the corresponding thiols and sulfo acids. *In the latter pair the change in the polarity of the significant group was brought about without substitution. Thus the standard for comparison was the substance in which a Walden inversion could not have occurred.* In the aliphatic series the oxidation of the sulfhydryl group was uniformly accompanied with a change of direction of rotation. On the basis of this behavior it was concluded that the configurations of the carbinols and of the halides were similar when the substances rotated in opposite directions.

The present investigation deals with the derivatives of optically active disubstituted acetic acids of the type.



[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **75**, 587 (1927).

The asymmetric carbon atom was not involved in any one of the series of transformations performed on the parent substance as is readily seen from the structure of the derivatives given in the following figures.



The derivatives of four acids were prepared by us, namely *n*-propyl-, *n*-butyl-, *n*-heptyl-, and *n*-decylmethylacetic acids. In addition there were prepared the mercaptan and the sulfo derivatives of optically active primary amyl alcohol. The other derivatives of this series are already known. Thus, the derivatives of five substances were made the subject of our present scrutiny.

The results of the observations are summarized in Table I. From Table I it is seen that in every instance the halide rotates in the opposite direction from that of the carbinol. The case of the thiol and sulfo derivatives needs special discussion. These two substances rotate in the same direction, whereas the secondary mercaptans on oxidation to the sulfo derivatives change the direction of their rotation. However, if the numerical values of the two substances are compared, it is found that the sulfo compound has the higher dextrorotation in the same manner as in the pair, carbinol and halide, the halide possessing the higher dextrorotation. In other words, the change of rotation on passing from the carbinol to the halide is in the same direction as on passing from the thiol to the sulfo derivative.

Thus the conclusion that in the aliphatic series the carbinols and

the related halides rotate in opposite directions is substantiated by the second method.

The long series of derivatives obtained in this work furnishes good material for more comprehensive speculations on the theme of the influence of individual substituting groups on the direction and on the magnitude of the optical rotations.

The influence of the substitution as was already mentioned was similar for all the analyzed substances. It is seen from Table I that starting with dextro-nitrile, the rotation gradually declines changing to the opposite direction for the amines and for the carbinols. Thus, there is a decided uniformity of the effect of each substituting group depending upon its respective polarity, or, in other words, upon its position in the polarity series.

$$C \equiv N > COOC_2H_5 > COOH > CONH_2 > (COCl) > CH_2SO_3H > CH_2X > CH_2SH > CH_2OH > CH_2NH_2$$

Chemists in recent years have devoted much attention to the respective polarities of substituting groups and have arranged them in certain series. The respective positions of individual groups in the different systems differed according to the method which had been employed for the rating of the respective polarities. The following have been used as the basis of rating: First, their directive influences on the substitution in the benzene nucleus.[2] According to this system, the polarities may be arranged in the following way: $\overline{OH}$, F, Cl, I, Br, $\overline{CH_3}$; $\overset{+}{H}$, CO_2H, $\overset{+}{NO_2}$. Second, the influence of the group on the dissociation constants of the substituted acids. The following series is then obtained: $\overline{Cl}$, Br, OCH_3, OH, $\overline{CH_3}$; H, $CO_2\overset{+}{H}$, CN, $\overset{+}{NO_2}$. Third, the specific inductive capacities of the substituting groups. The following series is then obtained: $\overline{OH}$, Cl, Br, I, $\overline{CH_3}$; $\overset{+}{H}$, CO_2H, CHO, $COCH_3$, CN, $\overset{+}{NO_2}$.

Comparing the positions of the individual members in the series observed by us with that in the series of polarities based on the first or on the third principle, it is seen that our series coincides well with these polarity series. In fact, Rule already has pointed

[2] Rule, H. G., *J. Chem. Soc.*, **125**, 1121 (1924).

out such a similarity in regard to several derivatives of levo-amyl alcohol. *Thus, in the series of aliphatic substances which may be regarded as derivatives of disubstituted acetic acids formed by substitution of the carboxyl, or of the corresponding carbinols by the substitution of the alcoholic groups, by other groups, the direction of the rotation and the respective numerical values of the latter may be regarded as functions of the polarity of the substituting group.*

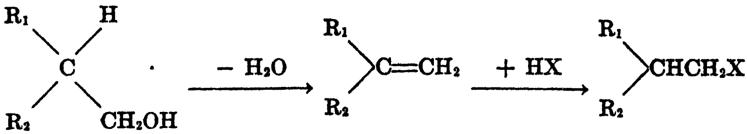
However, it should be emphasized that this simple relationship exists only in the series of aliphatic substances containing only one asymmetric carbon and only one polar group. The behavior of the substances with more than one polar group as well as of those containing an aromatic group is more complex and will be discussed in a later publication.

In conclusion we wish to mention some of the serious difficulties which were encountered in the course of the preparation of the substances described in the present publication. As was mentioned earlier in this paper, the principal task of the present undertaking was to compare the rotations of the carbinols and halides on one hand, and the thiol and sulfo derivatives on the other, of the series homologous to active primary amyl alcohol. *A priori*, no difficulty was anticipated in view of the fact that all chemical operations were performed on the terminal and not on the asymmetric carbon atom. To our surprise, we found that the conversion of the carbinol to the halide was invariably accompanied with complete racemization. The reagents were varied from $SOCl_2$ and PCl_5 to HBr and HI; also the temperatures and other conditions of reactions were varied in many ways but always without success. Therefore, it was concluded to resort to the action of nitrosyl chloride on the corresponding amines for the preparation of the chlorides. However, this reaction also was accompanied with complete racemization. Only after nitrosyl bromide was substituted for the chloride was it possible to obtain optically active halides.

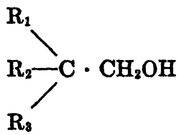
However, the optical activity of the halides was not sufficiently high to make the transformation of the bromides into the optically active mercaptans and into the sulfo derivatives promising.

It is worthy of note that the first member of this series of carbinols, namely the active primary amyl alcohol, was halogenated without marked racemization, so that it was readily transformed into the optically active thiol and sulfo derivatives.

Naturally, it is interesting to inquire into the mechanism of the racemization. The racemization resulting from the action of hydrogen halides may be explained on the assumption of the formation of an intermediary unsaturated compound in the following manner.



We intended to test this possibility by studying the conduct of carbinols with a tertiary carbon atom of the general formula of



Unfortunately, so far we have not succeeded in the preparation of these alcohols. It is significant that the amyl alcohol in the transformation of which racemization does not take place possesses the least asymmetry inasmuch as the two groups methyl and ethyl differ only by one CH_2 group and the groups ethyl and oxymethyl have practically the same molecular weight.[3]

EXPERIMENTAL.

Resolution of n-Propylmethylacetic Acid.—The acid was converted into the quinine salt in 50 per cent acetone solution. It was then recrystallized from 50 per cent acetone until further recrystallizations no longer increased the activity. The salt was then decomposed with hydrochloric acid and the organic acid extracted with ether, washed, and finally dried over anhydrous sodium sulfate. The ether was removed and the acid distilled at a pressure of 4 mm. It distilled at 83–84° and showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.66^\circ \times 100}{1 \times 20.424} = -8.13^\circ. \quad [M]_D^{25} = -9.42^\circ \text{ (in ether)},$$

[3] We wish to acknowledge the assistance of Dr. Kurt Passoth and Mr. R. E. Marker in the preparation of several substances employed in this investigation.

$$[\alpha]_D^{25} = \frac{-1.24° \times 100}{1 \times 15.944} = -7.78°. \quad [M]_D^{25} = -9.01° \text{ (in 50 per cent alcohol).}$$

$$[\alpha]_D^{25} = -7.71°. \qquad [M]_D^{25} = -8.91° \text{ (without solvent).}$$

0.3658 gm. of the above acid was treated with 1.5 cc. of 1.97 N NaOH; the solution was diluted to 2.5 cc. with water. The rotation in a 1 dm. tube was found to be —0.64°.

$$[\alpha]_D^{25} = \frac{-0.64° \times 100}{1 \times 17.40} = -3.68°. \quad [M]_D^{25} = -5.06° \text{ (in water).}$$

Dextro-n-Propylmethylacetyl Chloride.—50 gm. of dextro-*n*-propylmethylacetic acid

$$[\alpha]_D^{25} = \frac{+1.04° \times 100}{1 \times 18.640} = +5.58°. \quad [M]_D^{25} = +6.47° \text{ (in ether)}$$

were poured slowly with cooling into 200 cc. of thionyl chloride. After all the acid had been added the mixture was refluxed on the steam bath for one-half hour. It was then fractionated under a pressure of 15 mm. The fraction distilling at 45–48° showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.34° \times 100}{1 \times 8.224} = +4.13°. \quad [M]_D^{25} = +5.55° \text{ (in ether).}$$

$$[\alpha]_D^{25} = +4.06°. \qquad [M]_D^{25} = +5.46° \text{ (without solvent).}$$

0.1088 gm. substance: 7.70 cc. 0.1 N $AgNO_3$ (titration).
$C_6H_{11}OCl$. Calculated. Cl 26.39. Found. Cl 25.12.

On regeneration of the acid from the acid chloride, the rotation of this acid was found to be unchanged. Hence obviously no racemization had taken place during the process of chlorination.

Dextro-n-Propylmethylacetamide.—60 gm. of dextro-*n*-propylmethylacetyl chloride

$$[\alpha]_D^{25} = \frac{+0.56° \times 100}{1 \times 14.220} = +3.94°. \quad [M]_D^{25} = +5.30° \text{ (in ether)}$$

were dropped slowly with rapid stirring and thorough cooling into 250 cc. of concentrated aqueous ammonia. The amide was filtered

off and recrystallized from water until free of ammonium chloride. It melted at 78° and showed the following rotation.

$$[\alpha]_D^{25} = \frac{+0.47° \times 100}{1 \times 8.120} = +5.79°. \quad [M]_D^{25} = +6.65° \text{ (in 75 per cent alcohol).}$$

0.1000 gm. substance: 8.50 cc. 0.1 N HCl (Kjeldahl).
$C_6H_{13}ON$. Calculated. N 12.17. Found. N 11.90.

Levo-n-Propylmethylacetonitrile.—20 gm. of the corresponding amide

$$[\alpha]_D^{25} = \frac{-0.42° \times 100}{1 \times 7.258} = -5.79°. \quad [M]_D^{25} = -6.64° \text{ (in 75 per cent alcohol)}$$

were distilled with 1 molecule of phosphorus pentoxide under a pressure of about 2 mm. The distillate was dissolved in ether, washed with water, and dried over anhydrous sodium sulfate. The ether was removed under atmospheric pressure and the residue fractionated under a pressure of 2 mm. The nitrile distilled at 30–32°. The yield was good.

$$[\alpha]_D^{25} = \frac{-1.70° \times 100}{1 \times 11.241} = -15.12°. \quad [M]_D^{25} = -14.65° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-1.44° \times 100}{1 \times 10.204} = -14.11°. \quad [M]_D^{25} = -13.68° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = -13.77°. \qquad [M]_D^{25} = -13.35° \text{ (without solvent).}$$

4.370 mg. substance: 11.865 mg. CO_2 and 4.295 mg. H_2O.
$C_6H_{11}N$. Calculated. C 74.15, H 11.43.
Found. " 74.04, " 10.99.

Dextro-2,2-n-Propylmethylethylamine.—6.5 gm. of the above levo-*n*-propylmethylacetonitrile were dissolved in 150 cc. of alcohol which had been previously distilled over sodium. 21.5 gm. (16 mol) of sodium were then added to the boiling solution with rapid stirring. The mixture was then cooled and acidified with an excess of hydrochloric acid. The alcohol was distilled off under reduced pressure and the solution was extracted with ether to remove traces of unchanged nitrile. Excess of alkali was then added and the amine was extracted with ether, washed, and dried

over anhydrous sodium sulfate. The residue was distilled under a pressure of 4 mm. The fraction distilling at 28–30° showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.48° \times 100}{1 \times 16.756} = +2.86°. \quad [M]_D^{25} = +2.89° \text{ (in 50 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{+0.54° \times 100}{1 \times 13.112} = +4.12°. \quad [M]_D^{25} = +4.15° \text{ (in ether).}$$

$$[\alpha]_D^{25} = +3.84°. \qquad [M]_D^{25} = +3.86° \text{ (without solvent).}$$

0.932 gm. substance: 8.28 cc. 0.1 N HCl (Kjeldahl).
$C_6H_{15}N$. Calculated. N 13.84. Found. N 12.73.

Some of the above amine was converted to the hydrochloride in dry ether. The precipitate was merely washed with ether and dried. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.18° \times 100}{1 \times 11.94} = +1.51°. \quad [M]_D^{25} = +2.06° \text{ (in 50 per cent alcohol).}$$

0.1000 gm. substance: 7.25 cc. 0.1 N HCl (Kjeldahl).
0.1000 " " : 7.13 " 0.1 " $AgNO_3$ (titration).
$C_6H_{16}NCl$. Calculated. N 10.19, Cl 25.81.
Found. " 10.15, " 25.31.

Levo-n-Propylmethylacetic Acid Ethyl Ester.—3 gm. of levo-*n*-propylmethylacetic acid

$$[\alpha]_D^{25} = \frac{-0.89° \times 100}{1 \times 12.584} = -7.08°. \quad [M]_D^{25} = -8.21° \text{ (in ether);}$$

$$[\alpha]_D^{25} = \frac{-0.78° \times 100}{1 \times 12.624} = -6.18°. \quad [M]_D^{25} = -7.16° \text{ (in 75 per cent alcohol)}$$

were dissolved in 25 cc. of dry alcohol. The solution was saturated with HCl gas at −10° and allowed to stand at 0° for 24 hours. It was then poured on crushed ice, diluted with water and extracted with ether. The extract was washed first with dilute sodium hydroxide, then with water, and finally dried over anhydrous sodium sulfate. On removal of the ether, the residue was fractionated under a pressure of 4 mm. The ester distilled at 78–80°. Total yield 3.5 gm.

It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.08° \times 100}{13.600 \times 1} = -7.93°. \quad [M]_D^{25} = -11.43° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-1.32° \times 100}{1 \times 16\ 328} = -8.09°. \quad [M]_D^{25} = -11.63° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = -7.91°. \quad [M]_D^{25} = -11.39° \text{ (without solvent).}$$

Levo-2,2-n-Propylmethylethanol.—15 gm. of dextro-*n*-propylmethylacetic acid ethyl ester,

$$[\alpha]_D^{25} = \frac{+1.01° \times 100}{17.972 \times 1} = +5.67°. \quad [M]_D^{25} = +8.16° \text{ (in ether)}$$

were dissolved in 75 cc. of dry alcohol and allowed to drop slowly into an emulsion of 15 gm. of molten sodium in 75 cc. of dry toluene.[4] When all the sodium was dissolved the mixture was treated with water and extracted with ether. The extract was washed with water and dried over anhydrous sodium sulfate. The ether was then removed and the residue fractionated under atmospheric pressure. The fraction boiling at 147–147.5° weighed 12.5 gm. and showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.26° \times 100}{1 \times 32.472} = -0.80°. \quad [M]_D^{25} = -0.82° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-0\ 23° \times 100}{21\ 460 \times 1} = -1.07°. \quad [M]_D^{25} = -1.11° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = -1.23°. \quad [M]_D^{25} = -1.25° \text{ (without solvent).}$$

4 553 mg. substance: 11.758 mg CO_2 and 5.705 mg. H_2O.

$C_6H_{14}O$.	Calculated.	C 70 58,	H 13.88.
	Found.	" 70.42,	" 14.02.

Inactive 2,2-n-Propylmethylethyl Chloride.—3 gm. of levo-2,2-*n*-propylmethylethanol were added slowly with cooling to a suspension of 6 gm. of phosphorus pentachloride in chloroform. The mixture was allowed to stand at 0° for 2 hours, at the end of which time practically all the pentachloride had dissolved. The

[4] See Levene, P. A., and Allen, C. H., *J. Biol. Chem*, **27**, 433 (1916).

chloroform was then removed under reduced pressure. The residue was then shaken with water and extracted with ether. The extract was washed first with water then with dilute sodium hydroxide solution and then again with water. It was dried over anhydrous sodium sulfate and then distilled under atmospheric pressure. The substance distilled between 110-120°. The product was optically inactive.

0.1000 gm. substance: 0.1170 gm. AgCl (Carius).
$C_6H_{13}Cl$. Calculated. Cl 29.43. Found. Cl 28.94.

The action of nitrosyl chloride in ether solution on active 2,2-*n*-propylmethylethylamine also yielded an optically inactive halide.

Levo-2,2-n-Propylmethylethyl Bromide.—2 gm. of dextro-2,2-*n*-propylmethylethylamine, $[\alpha]_D^{25} = +4.12°$ (in ether), were dissolved in 20 cc. of concentrated hydrobromic acid, cooled to 0° and treated with 5 gm. of bromine. Nitric oxide was then passed through the solution for 4 hours. The solution was then diluted with water and extracted with ether. The extract was washed first with a little dilute sodium hydroxide and then with water. It was then dried over anhydrous sodium sulfate and finally distilled under a pressure of about 10 mm. The fraction distilling between 55–65° showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.47° \times 100}{1 \times 49.988} = -0.94°. \quad [M]_D^{25} = -1.51° \text{ (in ether)}.$$

0.1310 gm. substance: 0.1388 gm. AgBr (Carius).
$C_6H_{13}Br$. Calculated. Br 48.48. Found. Br 45.09.

Dextro-n-Propylmethylthiolacetic Acid.—10 gm. of dextro-*n*-propylmethylacetyl chloride

$$[\alpha]_D^{25} = \frac{+0.82° \times 100}{1 \times 21.072} = +3.89°. \quad [M]_D^{25} = +5.23° \text{ (in ether)}$$

were poured slowly with stirring and cooling into an aqueous solution of potassium hydrogen sulfide (12.51 gm. of KOH in 8 cc. of H_2O, saturated with H_2S). When the reaction mixture was taken out of the ice bath, the temperature gradually rose until it reached 60°. The solution was then heated on the steam bath for 2 minutes, whereupon it was cooled and acidified. The thiol acid

was extracted with ether, washed with water, and dried over anhydrous sodium sulfate. The ether was removed and the residue distilled under a pressure of 23 mm. It boiled at 71–72°, and showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.56° \times 100}{1 \times 20.836} = +7.49°. \quad [M]_D^{25} = +9.88° \text{ (in ether).}$$

0.1040 gm. substance: 0.1896 gm. $BaSO_4$ (Carius).

$C_6H_{12}OS$. Calculated. S 24.29. Found. S 25.05.

Resolution of n-Butylmethylacetic Acid.—112 gm. of racemic *n*-butylmethylacetic acid were dissolved in 750 cc. of 66 per cent acetone. To the warm solution were added 253 gm. of cinchonidine. On cooling and stirring the salt precipitated. The salt was then repeatedly recrystallized from 66 per cent acetone until the acid obtained on decomposition of a little of the salt showed a very slight increase of rotation. The salt was then decomposed with hydrochloric acid, the acid extracted with ether, washed, dried, and finally distilled under a pressure of 20 mm. It distilled at 121–122°. Yield 42 gm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-2.16° \times 100}{1 \times 14.164} = -15.25°. \quad [M]_D^{25} = -19.82° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-2.22° \times 100}{1 \times 18.432} = -12.04°. \quad [M]_D^{25} = -15.65° \text{ (in 95 per cent alcohol).}$$

$$[\alpha]_D^{25} = -14.56°. \qquad [M]_D^{25} = -18.91° \text{ (without solvent).}$$

0.5536 gm. of the above acid was treated with 1 cc. of 5 N NaOH (0.85 cc. = 1 mol). The solution was diluted to 2.5 cc. and rotation determined in a 1 dm. tube. The reading was −1.10°. Hence for the Na salt

$$[\alpha]_D^{25} = \frac{-1.10° \times 100}{1 \times 25.892} = -4.25°. \quad [M]_D^{25} = -6.47° \text{ (in water).}$$

Dextro-n-Butylmethylacetyl Chloride.—43 gm. of dextro-*n*-butylmethylacetic acid

$$[\alpha]_D^{25} = \frac{+0.98° \times 100}{1 \times 18.080} = +5.42°. \quad [M]_D^{25} = +7.04° \text{ (in ether);}$$

$$[\alpha]_D^{25} = \frac{+0.61° \times 100}{1 \times 13.824} = +4.41°. \quad [M]_D^{25} = +5.73° \text{ (in 75 per cent alcohol)}$$

were poured slowly with cooling into 100 gm. of thionyl chloride. The mixture was refluxed for $\frac{1}{2}$ hour under a return condenser and was then fractionated under a pressure of 9 mm. The chloride distilled at 45–48°, and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.76° \times 100}{1 \times 16.928} = +4.49°. \quad [M]_D^{25} = +6.66° \text{ (in ether).}$$

$$[\alpha]_D^{25} = +5.06°. \qquad [M]_D^{25} = +7.51° \text{ (without solvent).}$$

0.1386 gm. substance: 8.80 cc. 0.1 N $AgNO_3$ (titration).
$C_7H_{13}OCl$. Calculated. Cl 23.90. Found. Cl 22.54.

Dextro-n-Butylmethylacetamide.—20 gm. of the above dextro-*n*-butylmethylacetic acid chloride were added slowly with rapid stirring and thorough cooling to 100 cc. of concentrated aqueous ammonia. The amide crystallized at once. After two recrystallizations from water it was free from ammonium chloride. It melted at 66° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.58° \times 100}{1 \times 15.024} = +3.86°. \quad [M]_D^{25} = +4.98° \text{ (in 75 per cent alcohol).}$$

0.1000 gm. substance: 7.58 cc. 0.1 N HCl (Kjeldahl).
$C_7H_{15}ON$. Calculated. N 10.85. Found. N 10.61.

Dextro-n-Butylmethylacetonitrile.—18 gm. of dextro-*n*-butylmethylacetamide, $[\alpha]_D^{25} = +3.26°$ (in ether), were mixed with 20 gm. of phosphorus pentoxide. The mixture was then distilled under a pressure of 9 mm. The distillate was redistilled under the same pressure. The nitrile boiled at 43–50°, yielding 12 gm. of the purified substance, which showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.91° \times 100}{1 \times 9.936} = +9.16°. \quad [M]_D^{25} = +10.16° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.13° \times 100}{1 \times 11.242} = +10.05°. \quad [M]_D^{25} = +11.15° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +9.40°. \qquad [M]_D^{25} = +10.43° \text{ (without solvent).}$$

4.385 mg. substance: 12.125 mg. CO_2 and 4.610 mg. H_2O.
0.1246 gm. " : 10.70 cc. 0.1 N HCl (Kjeldahl).
$C_7H_{13}N$. Calculated. C 75.50, H 11.79, N 12.72.
Found. " 75.40, " 11.76, " 12.02.

In another experiment a nitrile was obtained with a rotation of

$$[\alpha]_D^{25} = \frac{-2.98^\circ \times 100}{1 \times 11.000} = -27.09^\circ.\quad [M]_D^{25} = -30.06^\circ \text{ (in ether);}$$

$$[\alpha]_D^{25} = \frac{-3.39^\circ \times 100}{1 \times 11.416} = -29.69^\circ.\quad [M]_D^{25} = -52.95^\circ \text{ (in 75 per cent alcohol)}$$

from an amide with a rotation of

$$[\alpha]_D^{25} = \frac{-2.52^\circ \times 100}{1 \times 22\,030} = -11.44^\circ.\quad [M]_D^{25} = -14.74^\circ \text{ (in 75 per cent alcohol).}$$

Levo-2, 2-n-Butylmethylethylamine.—5 gm. of dextro-*n*-butylmethylacetonitrile

$$[\alpha]_D^{25} = \frac{+0\,91^\circ \times 100}{1 \times 9\,936} = +9.16^\circ.\quad [M]_D^{25} = +10.16^\circ \text{ (in ether)}$$

were dissolved in 150 cc. of dry alcohol. The solution was heated to boiling whereupon 16.48 gm. of sodium were added in small amounts with rapid stirring to the refluxing solution. When all the sodium had dissolved the reaction mixture was cooled and acidified with a slight excess of hydrochloric acid. The alcohol, as well as most of the water, was distilled off under reduced pressure. The residue was treated with excess of sodium hydroxide, and the amine extracted with ether. The extract was washed and then dried over anhydrous sodium sulfate. The ether was removed and the residue fractionated under a pressure of 15 mm. The amine distilled at 49–54°, and showed a rotation of

$$[\alpha]_D^{25} = \frac{-0\,34^\circ \times 100}{1 \times 9.668} = -3.52^\circ.\quad [M]_D^{25} = -4.03^\circ \text{ (in ether).}$$

A little of the amine was dissolved in dry ether and precipitated with hydrochloric acid gas. The hydrochloride was dissolved in absolute alcohol and precipitated with ether. In aqueous solution the hydrochloride showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.37^\circ \times 100}{1 \times 15.384} = -2.41^\circ.\quad [M]_D^{25} = -3.63^\circ.$$

0.1000 gm substance: 6 42 gm 0 1 N HCl (Kjeldahl).
$C_7H_{18}NCl$. Calculated. N 9 24. Found. N 8.99.

Dextro-n-Butylmethylacetic Acid Ethyl Ester.—10 gm. of dextro-*n*-butylmethylacetic acid

$$[\alpha]_D^{25} = \frac{+1.04° \times 100}{1 \times 14.488} = +7.18°. \quad [M]_D^{25} = +9.32° \text{ (in ether)}$$

were dissolved in 50 cc. of absolute alcohol. The solution was then cooled to -10° and maintained below zero while it was being saturated with hydrogen chloride gas. It was then allowed to stand at 0° for 2 days; water was then added and the ester extracted with ether. The extract was washed free of hydrochloric acid and then dried over anhydrous sodium sulfate. The ether was removed and the residue distilled under a pressure of 9 mm. The substance boiled at 58–62°; yield 10.5 gm.

$$[\alpha]_D^{25} = \frac{+0.57° \times 100}{1 \times 7.480} = +7.62°. \quad [M]_D^{25} = +12.04° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{+0.34° \times 100}{1 \times 4.296} = +7.91°. \quad [M]_D^{25} = +12.49° \text{ (in ether).}$$

$$[\alpha]_D^{25} = +6.84°. \qquad [M]_D^{25} = +10.80° \text{ (without solvent).}$$

5.250 mg. substance: 13.175 mg. CO_2 and 5.425 mg. H_2O.

$C_9H_{18}O_2$. Calculated. C 68.31, H 11.44.

Found. " 68.43, " 11.56.

Dextro-2,2-n-Butylmethylethanol.—12.9 gm. of levo-*n*-butylmethylacetic acid ethyl ester

$$[\alpha]_D^{25} = \frac{-2.51° \times 100}{18.256 \times 1} = -13.75°. \quad [M]_D^{25} = -21.71° \text{ (in ether)}$$

were dissolved in 100 cc. of absolute alcohol. The reduction was carried out exactly as described in the preparation of levo-2,2-*n*-propylmethylethanol. The carbinol was distilled under a pressure of 15 mm. It distilled at 71–72°, and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.32° \times 100}{1 \times 19.144} = +1.67°. \quad [M]_D^{25} = +1.94° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{+0.54° \times 100}{1 \times 21.864} = +2.47°. \quad [M]_D^{25} = +2.86° \text{ (in ether).}$$

4.795 mg. substance: 12.670 mg. CO_2 and 5.825 mg. H_2O.
$C_7H_{16}O$. Calculated. C 72.41, H 13 79.
Found. " 72.05, " 13.59.

Resolution of n-Heptylmethylacetic Acid.—122 gm. of racemic *n*-heptylmethylacetic acid were dissolved in 600 cc. of 66 per cent acetone; 209 gm. of cinchonidine were then added to the warm solution. The latter was allowed to stand overnight at room temperature. Next day the precipitate was filtered off and redissolved in 400 cc. of 66 per cent acetone and again allowed to stand overnight at room temperature. After three recrystallizations the acid obtained from the salt showed a rotation of

$$[\alpha]_D^{25} = \frac{-2.38^\circ \times 100}{1 \times 25.352} = -9.39^\circ \text{ (in ether).}$$

In another experiment an acid was obtained which showed a rotation of

$$[\alpha]_D^{25} = \frac{-3.94^\circ \times 100}{29.95 \times 1} = -13.15^\circ \text{ (in ether).}$$

Still higher rotation could probably be obtained, but for the sake of conserving the material it was not recrystallized any further.

The acid obtained from the mother liquors was distilled under a pressure of 4 mm., under which pressure it boiled at 145–147°. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.67^\circ \times 100}{1 \times 18.232} = +9.16^\circ. \quad [M]_D^{25} = +15.75^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.19^\circ \times 100}{1 \times 15.880} = +7.49^\circ. \quad [M]_D^{25} = +12.88^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +8.34^\circ. \qquad [M]_D^{25} = +14.34^\circ \text{ (without solvent).}$$

To determine the rotation of the salt of the dextro acid described above, 0.3686 gm. of this acid was treated with 1.17 cc. of 1.97 N NaOH. The solution was diluted to 2.5 cc. In a 1 dm. tube the reading was +0.14°.

For the Na salt

$$[\alpha]_D^{25} = \frac{+0.14° \times 100}{1 \times 16.624} = +0.84°. \quad [M]_D^{25} = +1.63° \text{ (in water).}$$

Dextro-n-Heptylmethylacetyl Chloride.—80 gm. of dextro-*n*-heptylmethylacetic acid

$$[\alpha]_D^{25} = \frac{+1.91° \times 100}{1 \times 21.416} = +8.92°. \quad [M]_D^{25} = +15.34° \text{ (in ether)}$$

were poured slowly with cooling into 160 gm. of thionyl chloride. The mixture was then refluxed for ½ hour on the steam bath whereupon it was fractionated under a pressure of 1 mm. It distilled at 73–74° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.88° \times 100}{1 \times 17.512} = +5.02°. \quad [M]_D^{25} = +9.56° \text{ (in ether).}$$

$$[\alpha]_D^{25} = +4.89°. \qquad [M]_D^{25} = +9.31° \text{ (without solvent).}$$

0.1456 gm. substance: 7.50 cc. 0.1 N $AgNO_3$ (titration).
$C_{10}H_{19}OCl$. Calculated. Cl 18.63. Found. Cl 18.28.

Dextro-n-Heptylmethylacetamide.—The acid chloride described above was converted into the amide exactly as in the case of the corresponding *n*-butylmethyl compound. The amide obtained was repeatedly recrystallized from 50 per cent alcohol until it was free of chlorine ions. When pure it melted at 78° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.48° \times 100}{1 \times 6.792} = +7.07°. \quad [M]_D^{25} = +12.07° \text{ (in 95 per cent alcohol).}$$

0.1000 gm. substance: 5.56 gm. 0.1 N HCl (Kjeldahl).
$C_{10}H_{21}ON$. Calculated. N 8.18. Found. N 7.78.

Dextro-n-Heptylmethylacetonitrile.—50 gm. of the amide described in the previous experiment were mixed with 41.5 gm. of phosphorus pentoxide in a distilling flask and the nitrile was distilled off under a pressure of 7 mm. The distillate was redistilled under a pressure of 7 mm. The fraction boiling between 85–94° weighed 42 gm. and showed a rotation of

$$[\alpha]_D^{25} = \frac{+2.11° \times 100}{1 \times 14.312} = +14.74°. \quad [M]_D^{25} = +22.55° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.48° \times 100}{1 \times 9.824} = +15.07°. \quad [M]_D^{25} = +24.34° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +13.61°. \qquad [M]_D^{25} = +20.82° \text{ (without solvent).}$$

3.070 mg. substance: 8.835 mg. CO_2 and 3.330 mg. H_2O.
$C_{10}H_{19}N$. Calculated. C 78.38, H 12.47.
Found. " 78.47, " 12.13.

Levo-2,2-n-Heptylmethylethylamine.—43 gm. of the above dextro-*n*-heptylmethylacetonitrile were reduced with sodium in absolute alcohol exactly as in the case of the corresponding *n*-propylmethyl compound. The amine, of which 38 gm. were obtained, distilled at 103–105° under a pressure of about 25 mm. and showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.51° \times 100}{1 \times 18.280} = -2.79°. \quad [M]_D^{25} = -4.38° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{-0.54° \times 100}{1 \times 15.744} = -3.43°. \quad [M]_D^{25} = -5.38° \text{ (in ether).}$$

$$[\alpha]_D^{25} = -3.38°. \qquad [M]_D^{25} = -5.31° \text{ (without solvent).}$$

Some of the amine described above was dissolved in dry ether, and saturated with hydrobromic acid gas. The hydrobromide was immediately filtered off and the filtrate was then allowed to stand at room temperature. After a few hours standing a second crop of crystals was obtained. The first crop of crystals showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.65° \times 100}{1 \times 14.104} = -4.61°. \quad [M]_D^{25} = -10.97° \text{ (in 75 per cent alcohol).}$$

The second crop

$$[\alpha]_D^{25} = \frac{-0.7° \times 100}{1 \times 9.784} = -0.72°. \quad [M]_D^{25} = -1.68° \text{ (in 75 per cent alcohol).}$$

First Fraction.—0.1000 gm. substance: 4.30 cc. 0.1 N $AgNO_3$ (titration).
$C_{10}H_{24}NBr$. Calculated. Br 33.61. Found. Br 34.40.
Second Fraction.—0.1000 gm. substance: 4.20 cc. 0.1 N $AgNO_3$ (titration).
$C_{10}H_{24}NBr$. Calculated. Br 33.61. Found. Br 33.60.

That the reduction of the nitrile described above was accompanied with considerable racemization is shown by the fact that in another experiment a nitrile with a rotation of

$$[\alpha]_D^{25} = \frac{-1.88° \times 100}{1 \times 12.448} = -15.10°. \quad [M]_D^{25} = -23.10° \text{ (in ether)}$$

yielded an amine with a rotation of

$$[\alpha]_D^{25} = \frac{+1.19° \times 100}{1 \times 19.672} = +6.05°. \quad [M]_D^{25} = +9.48° \text{ (in ether).}$$

To determine the relationship between the rotatory power of the hydrobromide and the free amine, 0.3544 gm. of the hydrobromide with a rotation of

$$[\alpha]_D^{25} = \frac{+0.70° \times 100}{1 \times 11.848} = +5.91°. \quad [M]_D^{25} = +14.06° \text{ (in 75 per cent alcohol)}$$

was treated with 0.76 cc. of 1.97 N NaOH and the solution diluted to 5 cc. with absolute alcohol. Rotation in a 1 dm. tube was found to be +0.48°.

$$[\alpha]_D^{25} = \frac{+0.48° \times 100}{1 \times 4.670} = +10.28°. \quad [M]_D^{25} = +16.12°.$$

Dextro-2,2-n-Heptylmethylethyl Bromide.—5 gm. of levo-2,2-*n*-heptylmethylethylamine

$$[\alpha]_D^{25} = \frac{-1.08° \times 100}{1 \times 15.656} = -6.89°. \quad [M]_D^{25} = -10.81° \text{ (in 75 per cent alcohol)}$$

were dissolved in 30 cc. of fuming hydrobromic acid. The solution was thoroughly cooled and treated with sodium nitrite in slight excess. The acid solution was extracted with ether, the extract washed first with water, then with dilute sodium hydroxide, and then again with water. It was then dried over anhydrous sodium sulfate and finally distilled under a pressure of about 1 mm.; 4.5 gm. of the distillate boiling between 80–85° were obtained.

$$[\alpha]_D^{25} = \frac{+0.46° \times 100}{1 \times 21.096} = +2.18°. \quad [M]_D^{25} = +4.81° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.29° \times 100}{2 \times 5.034} = +2.88°. \quad [M]_D^{25} = +6.36° \text{ (in 90 per cent alcohol).}$$

0.1390 gm. substance: 0.1092 gm. AgBr (Carius).
$C_{10}H_{21}Br$. Calculated. Br 36.16. Found. Br 33.43.

Levo-n-Heptylmethylacetic Acid Ethyl Ester.—5 gm. of levo-*n*-heptylmethylacetic acid

$$[\alpha]_D^{25} = \frac{-1.56° \times 100}{1 \times 17.896} = -8.72°. \quad [M]_D^{25} = -14.99° \text{ (in ether)}$$

were dissolved in 15 cc. of dry alcohol, cooled in ice and salt mixture, and saturated with hydrochloric acid gas. The mixture was allowed to stand at 0° overnight, whereupon it was poured into water, extracted with ether, washed with dilute sodium hydroxide, then with water, and finally dried over anhydrous sodium sulfate. When dry, the ether was removed and the residue distilled under a pressure of 17 mm. The whole of the substance distilled at 122–124°; yield 6 gm.

$$[\alpha]_D^{25} = \frac{-0.99° \times 100}{1 \times 11.016} = -8.99°. \quad [M]_D^{25} = -17.94° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-1.26° \times 100}{1 \times 13.016} = -9.68°. \quad [M]_D^{25} = -19.36° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = -8.60°. \quad [M]_D^{25} = -17.20° \text{ (without solvent).}$$

4.755 mg. substance: 12.570 mg. CO_2 and 5.095 mg. H_2O.
$C_{12}H_{24}O_2$. Calculated. C 71.99, H 12.08.
Found. " 72.08, " 11.98.

Dextro-2,2-n-Heptylmethylethanol.—10 gm. of levo-*n*-heptylmethylacetic acid ethyl ester

$$[\alpha]_D^{25} = \frac{-1.01° \times 100}{1 \times 11.460} = -8.81°. \quad [M]_D^{25} = -17.62°$$

were dissolved in 50 cc. of dry alcohol. This was then added slowly with rapid stirring to an emulsion of 12 gm. of sodium in 75 cc. of boiling toluene. When all the ester had been added a little more alcohol was added to dissolve the excess sodium. The mixture was then cooled, water was added, and the alkaline solution was extracted with ether. The ether extract was washed

and dried and finally fractionated under a pressure of 0.4 mm. The carbinol distilled at 80–82°; yield 5 gm.

$$[\alpha]_D^{25} = \frac{+1.02° \times 100}{1 \times 23.904} = +4.27°. \quad [M]_D^{25} = +6.66° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.59° \times 100}{1 \times 14.280} = +4.13°. \quad [M]_D^{25} = +6.52° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +3.64°. \qquad [M]_D^{25} = +5.75° \text{ (without solvent).}$$

2.465 mg. substance: 6.875 mg. CO_2 and 3.095 mg. H_2O.

$C_{10}H_{22}O$.	Calculated.	C 75.84, H 13.98.
	Found.	" 76.05, " 14.04.

Resolution of n-Decylmethylacetic Acid.—The acid was prepared by means of the malonic ester synthesis. It was resolved as a cinchonidine salt by recrystallization from acetone containing about 5 per cent of water. The salt was repeatedly recrystallized from 95 per cent acetone until further recrystallization showed but slight increase in the activity of the acid obtained from the salt. Further resolution was then discontinued in order to conserve material. The acid distilled at 153° under a pressure of 1 mm. and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.77° \times 100}{1 \times 8.448} = +9.12°. \quad [M]_D^{25} = +19.51° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.62° \times 100}{1 \times 22.00} = +7.36°. \quad [M]_D^{25} = +15.72° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +8.47°. \qquad [M]_D^{25} = +18.12° \text{ (without solvent).}$$

The above acid when neutralized with 1 equivalent of sodium hydroxide showed no rotation whatever.

Levo-n-Decylmethylacetyl Chloride.—70 gm. of the levo acid

$$[\alpha]_D^{25} = -6.38°. \quad [M]_D^{25} = -13.65° \text{ (in ether)}$$

were treated with 140 gm. of thionyl chloride. The mixture was allowed to stand overnight at room temperature and was distilled the next day under a pressure of 0.5 mm. It distilled at 118–125° and showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.58^\circ \times 100}{1 \times 17.336} = -3.35^\circ. \quad [M]_D^{25} = -7.75^\circ \text{ (in ether)}.$$

$$[\alpha]_D^{25} = -3.5^\circ. \qquad [M]_D^{25} = -8.13^\circ \text{ (without solvent)}.$$

0.1230 gm. substance: 5.00 cc. 0.1 N $AgNO_3$ (titration).
$C_{13}H_{25}OCl$. Calculated. Cl 15.24. Found. Cl 14.43.

To determine whether any racemization had taken place during the reaction 4 gm. of the above acid chloride were treated with 20 cc. of H_2O and allowed to stand at room temperature overnight. The next day the acid was extracted with ether, washed, dried, and distilled under 0.7 mm. pressure. It distilled at 172–175° and showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.79^\circ \times 100}{1 \times 30.96} = -5.78^\circ. \quad [M]_D^{25} = -12.37^\circ \text{ (in ether)}.$$

Levo-n-Decylmethylacetamide.—70 gm. of the above acid chloride were dropped slowly with thorough cooling and rapid stirring into 400 cc. of concentrated ammonia. The amide was then filtered off. It was recrystallized from 50 per cent alcohol until free of ammonium chloride. When pure it melted at 77°, and showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.28^\circ \times 100}{1 \times 9.312} = -3.01^\circ. \quad [M]_D^{25} = -6.41^\circ \text{ (in 95 per cent alcohol)}.$$

3.445 mg. substance: 9.225 mg. CO_2 and 3.880 mg. H_2O.
$C_{13}H_{27}NO$. Calculated. C 73.23, H 12.76.
Found. " 73.02, " 12.60.

Levo-n-Decylmethylacetonitrile.—18 gm. of the above levo-*n*-decylmethylacetamide were treated with 18 gm. of P_2O_5 and distilled under a pressure of 0.5 mm. The distillate was then redistilled under the same pressure. It all distilled at 108–110°. It was levorotatory and showed a slightly higher rotation in alcohol than in ether, as shown from the figures below.

$$[\alpha]_D^{25} = \frac{-2.14^\circ \times 100}{1 \times 19.686} = -10.87^\circ. \quad [M]_D^{25} = -21.19^\circ \text{ (in ether)}.$$

$$[\alpha]_D^{25} = \frac{-1.97^\circ \times 100}{1 \times 17.864} = -11.03^\circ. \quad [M]_D^{25} = -21.51^\circ \text{ (in 95 per cent alcohol)}.$$

3.470 mg. substance: 10.200 mg. CO_2 and 3.890 mg. H_2O.
$C_{13}H_{25}N$. Calculated. C 80.00, H 12.92.
Found. " 80.15, " 12.54

Dextro-2,2-n-Decylmethylethylamine.—This amine was prepared from its nitrile by the same method which was used for the preparation of the other amines described in this paper. The nitrile from which the amine was prepared had a rotation of

$$[\alpha]_D^{25} = \frac{-2.14° \times 100}{1 \times 19.686} = -10.87°. \quad [M]_D^{25} = -21.19° \text{ (in ether)}.$$

The purified amine showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.78° \times 100}{1 \times 14.920} = +5.23°. \quad [M]_D^{25} = +10.41° \text{ (in ether)}.$$

$$[\alpha]_D^{25} = \frac{+0.60° \times 100}{1 \times 16.480} = +3.64°. \quad [M]_D^{25} = +7.23° \text{ (in 75 per cent alcohol)}.$$

$$[\alpha]_D^{25} = +4.18°. \quad [M]_D^{25} = +8.32° \text{ (without solvent)}.$$

3.130 mg. substance: 9.050 mg. CO_2 and 4.025 mg. H_2O.
$C_{13}H_{29}N$. Calculated. C 78.31, H 14.69.
Found. " 78.86, " 14.39.

2 gm. of the above amine were dissolved in 20 cc. of petrolic ether and saturated with hydrochloric acid gas. The hydrochloride was filtered off and washed with petrolic ether. No further purification was necessary. The hydrochloride softened at 105° and melted at 118°. Optical activity of the hydrochloride was determined.

$$[\alpha]_D^{25} = \frac{+0.19° \times 100}{1 \times 6.000} = +3.17°. \quad [M]_D^{25} = +7.46° \text{ (in water)}.$$

$$[\alpha]_D^{25} = \frac{+0.32° \times 100}{1 \times 10.112} = +3.16°. \quad [M]_D^{25} = +7.45° \text{ (in 75 per cent alcohol)}.$$

0.0991 gm. substance: 4.20 cc. 0.1 N $AgNO_3$ (titration).
$C_{13}H_{30}NCl$. Calculated. Cl 15.06. Found. Cl 15.04.

Levo-n-Decylmethylacetic Acid Ethyl Ester.—20 gm. of levo-*n*-decylmethylacetic acid

$$[\alpha]_D^{25} = \frac{-0.74° \times 100}{1 \times 12.560} = -5.89° \text{ (in ether)}.$$

were dissolved in 100 cc. of absolutely dry alcohol. The mixture was saturated with hydrochloric acid at —10° and allowed to stand at the room temperature for several days. Ice and water were then added and the ester extracted with ether. The extract was washed free from hydrochloric acid, dried over anhydrous sodium sulfate and finally distilled under a pressure of 1 mm. The entire substance, of which 21 gm. were obtained, distilled at 141°.

$$[\alpha]_D^{25} = \frac{-0.52° \times 100}{1 \times 8.552} = -6.08°. \quad [M]_D^{25} = -14.71° \text{ (in 87.5 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{-1.12° \times 100}{1 \times 19.00} = -5.89°. \quad [M]_D^{25} = -14.25° \text{ (in ether).}$$

$$[\alpha]_D^{25} = -6.48°. \quad [M]_D^{25} = -15.68° \text{ (without solvent).}$$

4.400 mg. substance: 12.00 mg. CO_2 and 5.055 mg. H_2O.
$C_{15}H_{30}O_2$. Calculated. C 74.30, H 12.65.
Found. " 74.37, " 12.85.

Levo-2, 2-n-Decylmethylethanol.—10 gm. of levo-*n*-decylmethylacetic acid ethyl ester

$$[\alpha]_D^{25} = \frac{-0.88° \times 100}{1 \times 15.336} = -5.74° \text{ (in ether)}$$

were dissolved in 50 cc. of dry alcohol and were dropped slowly, with rapid stirring, into a refluxing emulsion of 5.8 gm. of sodium in 75 cc. of dry toluene. When all the sodium was dissolved the mixture was cooled and treated with water. The mixture of the reduction product and toluene was extracted with ether, washed, and dried, and finally fractionated under a pressure of 1 mm. 20 gm. more of the ester were thus reduced and a total yield of 20 gm. of this reduction product was obtained. In order to purify the carbinol the reduction product was dissolved in 40 cc. of dry pyridine and treated with 14.8 gm. of phthalic anhydride. The mixture was allowed to stand at the room temperature for 48 hours. Ice and water were then added which was followed with excess of hydrochloric acid. The mixture was extracted with ether, and the extract washed with water. The ether was then removed and the residue dissolved in a slight excess of cold sodium hydroxide solution. The alkaline solution was extracted with ether to re-

move impurities. The aqueous layer was then acidified and extracted with chloroform and dried over anhydrous sodium sulfate. When dry the chloroform was removed and rotation on the residue determined.

$$[\alpha]_D^{25} = \frac{-0.07° \times 100}{1 \times 18.096} = -0.39°. \quad [M]_D^{25} = -1.34° \text{ (in ether)}.$$

The half ester was then decomposed by treating it with 3 mols of potassium hydroxide solution. The alcohol was extracted with ether, then dried and distilled under a pressure of 1.4 mm. It boiled at 105°.

$$[\alpha]_D^{25} = \frac{+0.51° \times 100}{1 \times 20.264} = +2.52°. \quad [M]_D^{25} = +5.02° \text{ (in ether)}.$$

$$[\alpha]_D^{25} = \frac{+0.48° \times 100}{1 \times 17.182} = +2.79°. \quad [M]_D^{25} = +5.58° \text{ (in 75 per cent alcohol)}.$$

$$[\alpha]_D^{25} = +2.34°. \quad [M]_D^{25} = +4.68° \text{ (without solvent)}.$$

3.965 mg. substance: 11.320 mg. CO_2 and 4.945 mg. H_2O.
$C_{13}H_{28}O$. Calculated. C 78.00, H 14.15.
Found. " 78.15, " 13.86

Levo-2,2-n-Decylmethylethyl Bromide.—10 gm. of dextro-2,2-*n*-decylmethylethylamine

$$[\alpha]_D^{25} = \frac{+0.78° \times 100}{1 \times 14.920} = +5.23°. \quad [M]_D^{25} = +10.41° \text{ (in ether)}$$

were dissolved in 50 cc. of dry ether and cooled to −55°. To this was then added a slight excess of ethereal solution of nitrosyl bromide. When the latter was no longer decolorized, water was added and the halide was extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. When the ether was removed the residue weighed 15 gm. and showed a rotation of

$$[\alpha]_D^{25} = -0.22°. \quad [M]_D^{25} = -0.58° \text{ (without solvent)}.$$

The small amount of carbinol which was present was removed by means of phthalic anhydride as described in previous experiments.

The halide was finally distilled under a pressure of 0.02 mm., b.p., 87–90°. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.06° \times 100}{1 \times 31.552} = -0.19°. \quad [M]_D^{25} = -0.31° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-0.09° \times 100}{2 \times 12.958} = -0.35°. \quad [M]_D^{25} = -0.55° \text{ (in 97.5 per cent alcohol).}$$

$$[\alpha]_D^{25} = -0.39°. \qquad [M]_D^{25} = -1.04° \text{ (without solvent).}$$

0.1014 gm. substance: 0.0662 gm. AgBr (Carius).

$C_{13}H_{27}Br$. Calculated. Br 30.41. Found. Br 27.78.

Dextro-2,2-Ethylmethylethyl Iodide.—120 gm. of primary levo-amyl alcohol

$$[\alpha]_D^{25} = \frac{-1.19° \times 100}{1 \times 25.156} = -4.73°. \quad [M]_D^{25} = -4.16° \text{ (in ether)}$$

were distilled with 360 gm. of constant boiling hydriodic acid. The distillate was diluted with water and extracted with ether. The extract was washed first with water, then with dilute sodium carbonate solution and then with water again. It was then dried over anhydrous sodium sulfate and finally fractionated. The halide distilled at 47–50° under a pressure of 22 mm.

$$[\alpha]_D^{25} = \frac{+1.18° \times 100}{1 \times 30.140} = +3.92°. \quad [M]_D^{25} = +7.74° \text{ (in ether).}$$

Dextro-2,2-Ethylmethylethanthiol.—40 gm. of the above described iodide were treated in a pressure bottle with 3 mols of alcoholic potassium hydrogen sulfide. The mixture was allowed to stand at room temperature for 2 days. Water was then added and the mercaptan extracted with ether. It was washed and dried and finally fractionated under atmospheric pressure. The mercaptan distilled at 116–117°.

$$[\alpha]_D^{25} = \frac{+0.31° \times 100}{1 \times 11.144} = +2.78°. \quad [M]_D^{25} = +2.9° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.48° \times 100}{1 \times 17.651} = +2.72°. \quad [M]_D^{25} = +2.88° \text{ (in 95 per cent alcohol).}$$

$$[\alpha]_D^{25} = +2.99°. \qquad [M]_D^{25} = +3.17° \text{ (without solvent).}$$

In another experiment an iodide with a rotation of $[\alpha]_D^{25} = +5.27°$ (in ether) yielded a mercaptan with a rotation of

$$[\alpha]_D^{25} = \frac{+1.55° \times 100}{1 \times 22.374} = +6.92°. \quad [M]_D^{25} = +7.19° \text{ (in ether).}$$

0.1023 gm. substance: 0.2292 gm. $BaSO_4$ (Carius).
$C_6H_{12}S$. Calculated. S 30.76. Found. S 30.78.

Dextro-2,2-Ethylmethylethanesulfonic Acid.—5 gm. of mercaptan, $[\alpha]_D^{25} = +6.92°$ (in ether), were dissolved in acetone containing about 5 per cent water. To this were then slowly added 18 gm. of barium permanganate dissolved in acetone. To complete the oxidation the mixture was heated for a short time on the steam bath. The manganese dioxide was filtered off and the solution was decolorized with another drop of the mercaptan. The filtrate was combined with the acetone and water washings of the manganese dioxide. The solution was then evaporated to dryness and the residue of barium salt was washed thoroughly with ether to remove ether-soluble impurities. The salt was recrystallized from water. It had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.78° \times 100}{1 \times 15.316} = +5.09°. \quad [M]_D^{25} = +11.17° \text{ (in water).}$$

0.0981 gm. substance: 0.0516 gm. $BaSO_4$ (for Ba) (Carius).
0.1134 " " : 0.1204 " " (" S) "
$C_{10}H_{22}S_2O_6Ba$. Calculated. Ba 31.54, S 14.59.
Found. " 30.95, " 14.58.

For the Ba salt in the presence of 2 equivalents of hydrochloric acid

$$[\alpha]_D^{25} = \frac{+0.64° \times 100}{1 \times 7.568} = +8.46°. \quad [M]_D^{25} = +12.84° \text{ (in water).}$$

Densities.

As the densities were required in the calculation of the molecular rotations without solvent, those not available in the literature were determined. They are all given in Table II.

TABLE I.

Molecular Rotations of Derivatives of Various Acids ($[M]_D^{25}$ *in Degrees*).*

Series of:	$-C(=O)-OH$	$-C(=O)-Cl$	$-C(=O)-SH$	$-C(=O)-OC_2H_5$	$-C(=O)-NH_2$	$-CN$	$-CH_2NH_2$	$-CH_2NH_2H_x$	$-CH_2x$	$-CH_2OH$	$-CH_2SH$	$-CH_2SO_3H$
n-Ethylmethylacetic acid.												
In ether..............									+10.43 (x=I)	−5.18	+7.19	
" 75 per cent alcohol..										−5.24	+6.78	+12.17 (acid)
Without solvent........	+19.03†			+22.87‡	+18.48§		−5.10†		+11.16† (x=I)	−4.78	+6.25	
In water..............												+12.84 (acid)
" "												+11.17 (Ba salt)
n-Propylmethylacetic acid.												
In ether..............	+7.29	+6.25	+10.64	+11 43		+14.58	−3.99		+1.41 (x=Br)	−1.92		
" 75 per cent alcohol..	+6.98		+10.87	+11.63	+6.65	+13 78	−2.88	−2.06 (x=Cl)		−1.48		
Without solvent........	+6 82	+4.58		+11.38		+13.35	−3.69			−1.66		
n-Butylmethylacetic acid.												
In ether..............	+7.04	+6.66		+9.44		+10.16	−4.03			−1.26		
" 75 per cent alcohol..	+5.73			+9.10	+4.98	+11.15		−3.63 (x=Cl)		−1.07		
Without solvent........	+6.31	+7.51		+8.16		+10.43						

TABLE I—*Concluded.*

Series of:	$-C(=O)OH$	$-C(=O)Cl$	$-C(=O)SH$	$-C(=O)OC_2H_5$	$-C(=O)NH_2$	$-CN$	$-CH_2NH_2$	$-CH_2NH_2Hx$	$-CH_2x$	$-CH_2OH$	$-CH_2SH$	$-CH_2SO_3H$
n-Heptylmethylacetic acid.												
In ether........	+15 94	+9 56		+18 23		+22 55	−5 38		+2 22	−6 74		
" 75 per cent alcohol.	+11 97			+18 64	+12 07	+24 34	−4 38	−2 99 (x=Br)	+2.93 (x=Br)	−6 85		
Without solvent.	+14 18	+10 41		+17 59		+20 84	−5 26			−5 95		
n-Decylmethylacetic acid.												
In ether.	+13 65	+7 76		+15 58		+21 19	−10 40		+0 50	−6 22		
" 75 per cent alcohol..	+11 00			+16 02	+6 38	+21 50	−7.24	−7 68 (x=Cl)	+0 89	−6.97		
Without solvent........	+12 67	+8 13		+17 00			−8 31		+0.97 (x=Br)	−3 43		

* It will be noted that the values of the rotations in this table do not always coincide with the rotations given in the text. The values in the table, in such cases, are extrapolated to the value of the acids given in this table. For instance, if the amine was prepared from a nitrile with a lower value than that obtainable from the highest acid, its rotation was recalculated on the basis of the parent substance with the higher rotation.

† As calculated from data found in Marckwald, W., *Ber. chem. Ges.*, **37**, 1045, 1048 (1904).

‡ Guye, P. A., *Bull. Soc. chim.*, **25**, series 3, 550 (1901).

§ $[M]_D^{17}$, as calculated with data of Taverne, H. J., *Rec. trav. chim. Pays-Bas*, **19**, 109 (1900).

TABLE II.

*Densities at 25° of Derivatives of Various Acids.**

Series of:	$—C(=O)OH$	$—C(=O)Cl$	$—C(=O)OC_2H_5$	$—CN$	$—CH_2NH_2$	$—CH_2x$	$—CH_2OH$	$—CH_2SH$
n-Ethylmethyl-acetic acid	0 938†						0 833†	0 848†
n-Propylmethyl-acetic acid.	0 928†	0 963	0 867†	0 791	0 763		0 824†	
n-Butylmethyl-acetic acid.	0 909	0 952	0 860	0 797				
n-Heptylmethyl-acetic acid.	0 893	0 894	0 856	0 809	0 789	1 082 (Br)	0 833†	
n-Decylmethyl-acetic acid.	0 884	0 908	0 854		0 806	1 168 (Br)	0 844	

* These values were used in calculating the specific and molecular rotations without solvent of the compounds recorded in the experimental part and in Table I.

† These values were found in the literature.

A MODIFIED VAN SLYKE AMINO NITROGEN APPARATUS.

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(Received for publication, August 23, 1929.)

The excellent amino nitrogen method of Van Slyke (1–3) and the unique apparatus devised by him for that purpose have contributed materially toward tremendous advances in numerous biochemical problems. However, in connection with my experience in teaching this method to others, I have observed a number of difficulties of manipulation and several sources of error. The defects I have in mind are the relatively crude mode of measuring the solution to be analyzed, the frequent loss of gas through the L-bore stop-cock connecting this measuring device with the reaction chamber, the tendency for gas to remain in the lower side arm of the reaction chamber, and the danger of losing some of the gas which always accumulates in the upright connecting tube below the stop-cock of the reservoir used for the storage of excess reagent.

The modified form here described overcomes these difficulties.[1] The special Ostwald pipette mounted on the L-bore stop-cock permits the more accurate measurement of 1 or 2 ml. quantities of the solution to be analyzed and the type of stop-cock attached thereto is also less likely to leak than the type formerly used. It is of course very essential that the solution to be analyzed be measured to the same degree of accuracy as the final volume of nitrogen collected. It may be recalled that in the regular micro form now available the gas burette is graduated to 0.01 ml. whereas the tube for measuring the solution to be analyzed does

[1] In connection with the construction of this apparatus I wish to acknowledge the heartiest cooperation of Mr. S. L. Redman of the Central Scientific Company of Chicago.

not permit of more accurate measurement than to 0.10 ml., although the order of total volumes is the same.

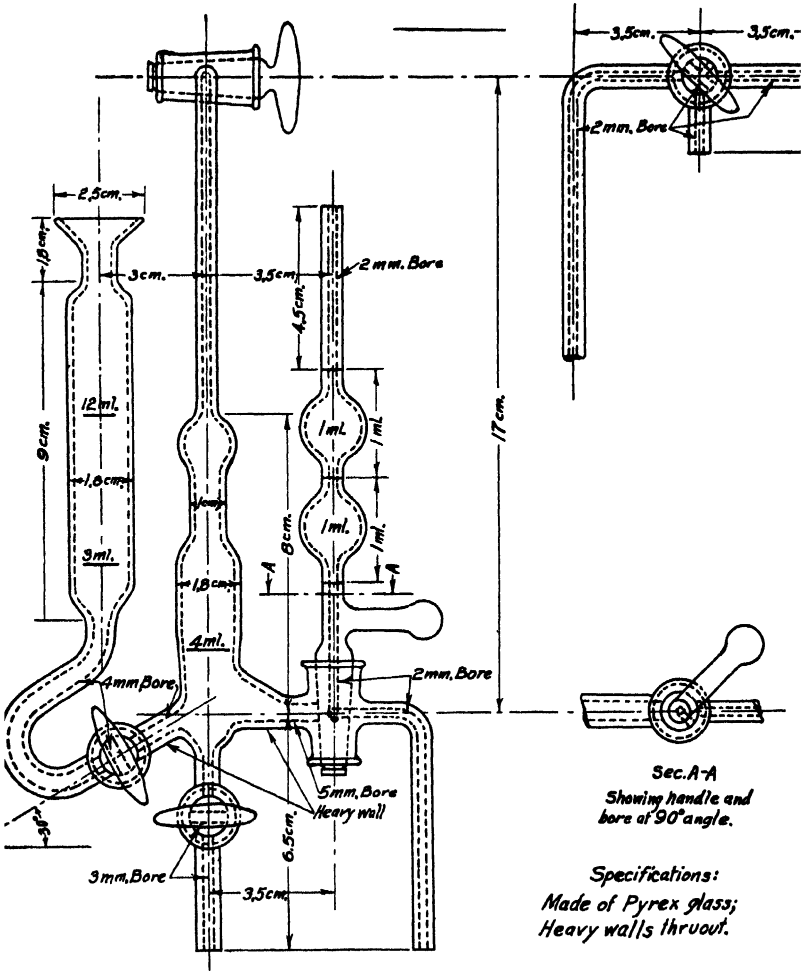

FIG. 1. Modified form of the Van Slyke amino nitrogen apparatus.

In the new form here shown (Fig. 1) the tube used for measuring and introducing 3 ml. of glacial acetic acid and 12 ml. of 30 per cent sodium nitrite solution is purposely placed lower and the stop-cock for connecting the same with the reaction chamber is

so located that there never can be an accumulation of gas in this part of the apparatus. It is a common experience in the hands of some to lose part of such accumulated gas in the old type of apparatus when trying to displace the gas by the excess reagent stored in the side tube for that purpose. The procedure to be followed in the modification here illustrated is essentially the same as in the original Van Slyke form. The only difference is that in the transfer of the glacial acetic acid and the sodium nitrite into the reaction chamber it is necessary to apply suction through the gas burette by lowering the levelling bulb instead of allowing these reagents to flow in by gravity. In the process of displacing and washing out the air in the reaction chamber it is also necessary to use the levelling bulb in the same way. After the reaction chamber has been freed from air one prepares the burette and the connecting capillary stop-cock in the usual way by displacing the gas therein with water from the burette. From here on the manipulation of the apparatus is the same as before.

Instead of using a special side tube so often attached for introducing caprylic alcohol to prevent foaming we have found it very satisfactory to omit this complication and to add to our glacial acetic acid, just before the introduction thereof, a small amount of caprylic alcohol. The addition of 0.5 to 1 per cent caprylic alcohol, shortly before use, to the glacial acetic acid is ample depending upon the nature of the substance analyzed.

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THE USE OF THE ANTIMONY-ANTIMONY TRIOXIDE ELECTRODE FOR DETERMINING THE DISSOCIATION CONSTANTS OF CERTAIN LOCAL ANESTHETICS AND RELATED COMPOUNDS.

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In an earlier paper (1) the antimony-antimony trioxide electrode and its use as a measure of acidity has been described in considerable detail. In this paper the discussion of the field of its applicability is extended to solutions of special interest to the biological chemist and pharmacologist, in which the use of the hydrogen electrode may be attended with difficulty or may yield conspicuously erroneous results. From the small amount of work that has been done on the subject it appears that an investigation of the acidity of solutions of local anesthetics may possibly provide a valuable measure of anesthetic efficiency, particularly of the relative efficiency of the members of a related series, and that it may in any case be a useful supplement to pharmacological methods. Therefore, four related local anesthetics belonging to the naphthalene series and first made in this laboratory (2) were selected, the pH of several concentrations of each anesthetic measured with the antimony electrode, and the dissociation constants of the four corresponding bases calculated by an extrapolation method. Particular emphasis has been laid on this series; but in order to be able to compare the constants so obtained with those published by other authors several better known compounds of similar structure, *i.e.* procaine, aniline hydrochloride, and diethylaminoethanol hydrochloride, were similarly investigated. The acidity of at least one dilution of each substance was determined with the hydrogen electrode as well as with the antimony electrode for the sake of comparison.

Dissociation Constant of a Weak Base from the Measured pH of Solutions of Its Hydrochloride.

Practically all the substances included in this investigation are the hydrochlorides of weak, relatively insoluble amino bases, a fact which precludes the use of titration methods, and necessitates operation through hydrolysis. Since dilute, or moderately dilute, solutions were used, complete dissociation of the salt may be assumed, together with unit activity of the water involved. Symbolically:

$B^+ + Cl^- + HOH \rightleftharpoons BOH + Cl^- + H^+$. At equilibrium

$$\frac{\alpha_{B^+}\,\alpha_{HOH}}{\alpha_{BOH}\,\alpha_{H^+}} = K = \frac{K_b}{K_w} = \frac{\alpha_{B^+}}{\alpha_{BOH}\,\alpha_{H^+}} \tag{1}$$

where α represents the activity of the substance designated and the equilibrium constant, K, is equal to the ratio of the dissociation constant of the base, K_b, to that of pure water, K_w. Let h be the fraction of the salt (hydrochloride) hydrolyzed; Equation 1 becomes

$$\frac{\gamma_{B^+}\,(1-h)m}{\gamma_{BOH}\,hm\,\alpha_{H^+}} = \frac{K_b}{K_w} \tag{2}$$

where γ is the activity coefficient of the substance specified and m the total molality of the salt. It is clear that $\alpha_{H^+} = \gamma_{H^+}hm$ or

$h = \dfrac{\alpha_{H^+}}{\gamma_{H^+}m}$, from which it follows that

$$\frac{\gamma_{B^+}\,(\gamma_{H^+}\,m - \alpha_{H^+})}{\gamma_{BOH}\,\alpha^2_{H^+}} = \frac{K_b}{K_w} \quad \text{and} \quad \frac{\gamma_{H^+}\,m - \alpha_{H^+}}{\alpha^2_{H^+}} \cdot \frac{\gamma_{B^+}}{\gamma_{BOH}} = \frac{K_b}{K_w} \tag{3}$$

α_{H^+} is so small as compared with $\gamma_{H^+}m$ that it may be omitted from our calculations and Equation 3 written

$$\log \gamma_{H^+}\,m + \log \gamma_{B^+} - \log \gamma_{BOH} + 2\,\text{pH} = \log \frac{K_b}{K_w} \tag{4}$$

From this it appears that the dissociation constant of the base may be calculated from the measured acidities of a series of solu-

tions of the salt, with the aid of certain assumptions. The error involved in putting γ_{H^+} equal to its value in solutions of pure hydrochloric acid of the same concentration is probably well within our experimental accuracy (3); values of log $\gamma_{H^+}m$ derived from the work of Scatchard (4) on the activity coefficients of potassium chloride and hydrochloric acid were used in our calculations, and are tabulated in the last column of Section A, Table I. The customary assumption that the activity coefficient of the

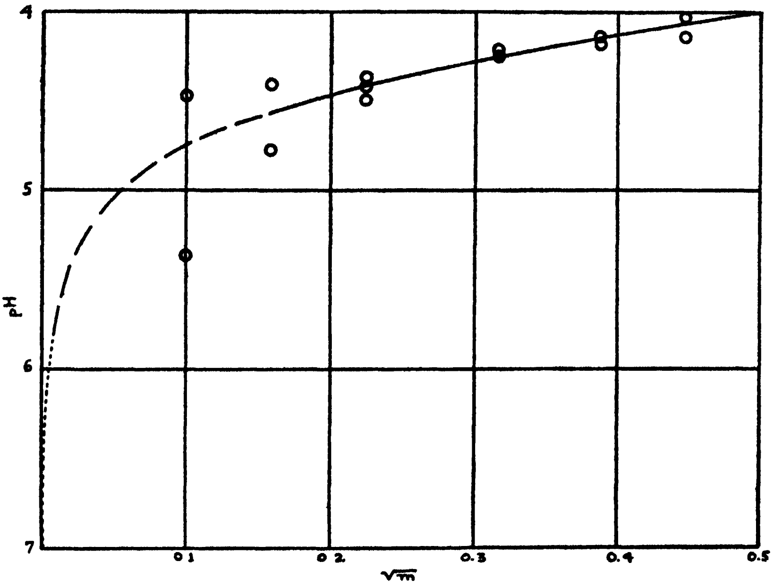


FIG. 1. The measured pH at different concentrations of the hydrochloride of diethylaminoethyl-β-naphthoate (Sample E.E.).

undissociated part of a weak electrolyte, here BOH, is unity, may be adopted. In dilute solution, according to the theory of Debye and Hückel, log $\gamma_{B^+} = -0.5\, m^{\frac{1}{2}}$. This is rigorously true only as m approaches 0 but, within our experimental error, holds over a considerable range in concentration.

Our procedure was as follows: The values of pH determined experimentally were plotted against the square root of the corresponding molalities and the "best curve" drawn, the slope being

fixed in dilute solution by Equation 4. Fig. 1 shows a typical curve and its relation to our assumed ideal. The region over which the theoretical curve fits the experimental data as well as any is indicated by a broken line; the calculations were not carried beyond 1 millimolal; the dotted continuation of the lower end of the curve merely indicates the rapid increase in pH as the solution approaches infinite dilution. At concentrations above the region of ideal slope a full line shows the course of the curve. From this curve values of pH at rounded molalities were read and the right hand member of Equation 4 calculated for each chosen concentration; these, in turn, were plotted against $m^{\frac{1}{2}}$. It is obvious that over the range in which the ideal slope fits the first plot, the second is a straight line parallel to the abscissa, its intersection with the ordinate giving the true value of $\log \frac{K_b}{K_w}$. Beyond the region of ideality, which is different in magnitude with different substances, the calculable value of K_b is only the approximate K'_b which may differ appreciably from the extrapolated value. The danger of making a serious error in computing the dissociation constant from a determination of the pH at a single dilution is evident. This method of extrapolation is not new; it has been used in a number of slightly varied forms, the one most closely approaching our own being that of Cohn, Heyroth, and Menkin (5) for determining the dissociation constant of acetic acid.

The most commonly used expression for $\frac{K_b}{K_w}$ is

$$\log m + 2 \text{ pH} = \log \frac{K_b}{K_w} \tag{5}$$

or, in its more familiar form, $a_{H^+} = \sqrt{\left(\frac{K_w}{K_b}\right) m}$. In this equation equal activities of undissociated base and H^+ are assumed, together with an activity of B^+ equal to m, the total molality of the salt. These assumptions are at variance with the modern theories regarding the behavior of strong electrolytes; nevertheless we have generally recorded in our tables $\log \frac{K_b}{K_w}$ (termed $\frac{K''_b}{K_w}$ in

this case) thus calculated, in order to contrast these with our own values. The difference between our apparent log $\frac{K_b}{K_w}$, or log $\frac{K'_b}{K_w}$, and log $\frac{K''_b}{K_w}$ is constant for any given concentration and is equal to log $\gamma_{H^+}m$ + log γ_{B^+} − log m, or log $\gamma_{H^+}\,\gamma_{B^+}$. Fig. 2 illustrates this difference with procaine. Curves A and A′ are the curves obtained by plotting log $\frac{K'_l}{K_w}$ and log $\frac{K''_b}{K_w}$, respectively,

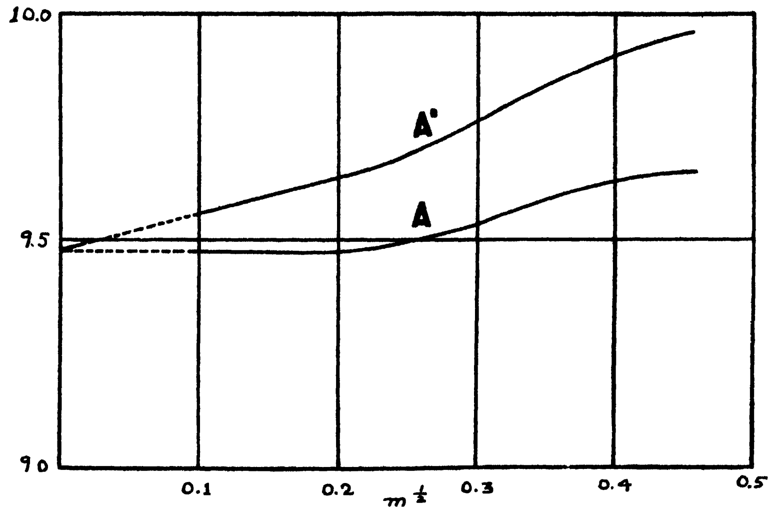


Fig. 2. The dissociation constant of procaine. Curve A, log $\frac{K'_b}{K_w}$ *versus* $m^{\frac{1}{2}}$. Curve A′, log $\frac{K''_b}{K_w}$ *versus* $m^{\frac{1}{2}}$.

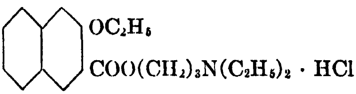
against $m^{\frac{1}{2}}$. The dotted lines in this, and also in Fig. 4, show the region in which no experimental determinations were possible. Curve A is more nearly parallel to the abscissa than Curve A′, which means that K'_b approaches K_b more closely than does K''_b. Further comparison of the two curves is pointless since both were calculated from the same pH *versus* $m^{\frac{1}{2}}$ plot and the assumptions made in fixing the slope of the latter in dilute solution affects both Curves A and A′. The slope of the pH curve

departs from the ideal near 50 millimolal; obviously if either curve were extrapolated only on the basis of the experimental data at concentrations greater than $m^{\frac{1}{2}} = 0.22$ a value for $\frac{K_b}{K_w}$ very different from the one indicated would be obtained.

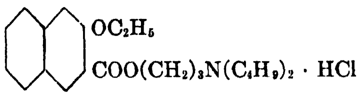
Preparation of Materials.

Anesthetics in the Naphthalene Series.—Four of the local anesthetics described by Hill and Robinson (2) were selected for this work; these authors will give in detail the preparation, purification, and analyses of the compounds chosen, which were:

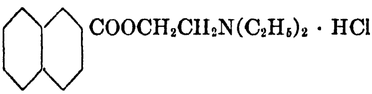
A. The hydrochloride of 2-ethoxy-3-diethylaminopropylnaphthoate (Sample E.P.).



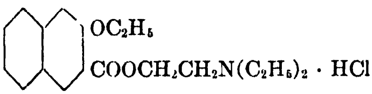
B. Hydrochloride of 2-ethoxy-3-dibutylaminopropylnaphthoate (Sample B.P.).



C. Hydrochloride of diethylaminoethyl-β-naphthoate (Sample βE.E.).



D. Hydrochloride of 2-ethoxy-3-diethylaminoethylnaphthoate (Sample E.E.)



Procaine (*Novocaine*). $NH_2\langle\qquad\rangle COOCH_2CH_2N(C_2H_5)_2\cdot HCl$.—A very pure sample of procaine, some of which had been used for research and standardization purposes, was generously given by the Metz Laboratories.

Diethylaminoethanol and Its Hydrochloride.—Commercial C.P. diethylaminoethanol was fractionally distilled six times until its boiling point did not change. B.p., 160–161°.

Some of this alcohol was dissolved in chloroform and dry hydrogen chloride passed in to saturation. The resulting hydrochloride was recrystallized several times from chloroform-acetone mixtures, when a beautifully crystalline product was obtained melting at 134–135°.

Calculated for $C_6H_{16}ONCl$. Cl 23.09.
Found. " 23.09.

Aniline Hydrochloride.—Aniline was distilled once through a long, efficient, fractionating column; the distillate had a constant boiling point of 183–183.5°. Dry hydrogen chloride was passed into its chloroform solution and the resulting hydrochloride recrystallized from absolute alcohol, chloroform being added to start crystallization. Several recrystallizations did not change the melting point; the salt was dried in a vacuum before use.

Benzoic Acid.—Benzoic acid was crystallized three times from alcohol-water mixtures and the resulting product fused and powdered before use.

p-Aminobenzoic Acid.—*p*-Aminobenzoic acid was recrystallized five times from boiling water, thrice with the use of norit. The resulting acid melted at 186°.

Description of Apparatus.

The form of antimony-antimony trioxide electrode used here has been described (1). It consists essentially of a column of crystals of pure antimony intimately mixed with cubic antimony trioxide, through which a solution of unknown acidity may be allowed to flow. The reference electrode, a silver-silver chloride electrode in 3.5 N potassium chloride, as well as the hydrogen electrode used in the latter part of the work, was also discussed in the earlier paper. All three of the electrodes were adapted for the use of a flowing junction during the time required for reading the potentiometer.

Since some of the substances included in this investigation show a serious tendency to salt out at the junction between the two electrodes, the perforated mica plate (6) previously used for the liquid junction was abandoned in most cases in favor of a strip of hardened filter paper coated with paraffin on both sides except for a section about 8 × 10 mm. interposed directly between the exit tubes of the two electrodes. A strip of paraffined paper pressed onto the plate near the bottom and bent slightly forward prevented the streaming electrolytes from converging. The paper plate is sketched in Fig. 3. The results obtained with this device were satisfactorily consistent; after four or five readings the plate was discarded and a new one substituted.

An air thermostat constant to ±0.05° kept the electrodes at 25°. The measurements were made with a Leeds and Northrup

type K potentiometer, the working standard Weston cell being compared at frequent intervals with two certificated cells of the unsaturated type purchased from different makers.

Experimental Procedure.

Solutions of the several organic compounds were made by dissolving a weighed amount of material in the measured volume of freshly boiled and cooled distilled water calculated to give the desired concentration. The antimony electrode, which was frequently pretreated with a solution of sodium hydroxide as recom-

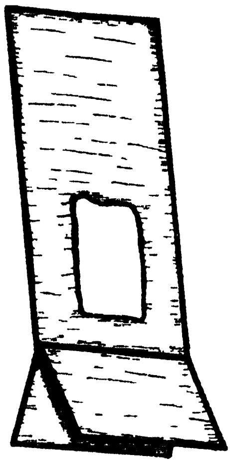


FIG 3. Sketch of paper plate for flowing junctions

mended (1), was carefully washed with water and then very thoroughly with small portions of the solution to be introduced, the reservoir filled, and a small soda-lime tube attached to prevent the absorption of carbon dioxide by the solution. Unless otherwise specifically stated all measurements were made in air; for this reason, and also because some solutions (discussed later) behaved abnormally, a standard buffer solution was measured in many cases immediately after each unknown with no intervening change of solution in the electrode. The potential difference between the antimony electrode and the standard silver chloride was read at intervals over a period of at least 24 hours, usually

several days. During this time the solution was not allowed to flow past the metal but only through the by-pass tube to the junction, except at the close of each working day, when the antimony crystals were rinsed with fresh electrolyte.

A potential time curve was plotted for each unknown and compared with a similar curve for a standard buffer of not widely different pH; the normal curve rises sharply at first, then gradually approaches a value which shows little change with time. Ordinarily 24 hours are more than sufficient time for the difference between the curve for the unknown and a suitable buffer to become reasonably constant, therefore this time served as a general reference datum.

Adopted Reference Values and Conventions.

The buffer solutions used for acidity standards in the antimony electrode were selected from those of the Clark and Lubs series, the pH values for which were fixed at 20° but have been corrected to 25° (1). In order that any error involved in making the assumption that the potential change of the antimony electrode per pH unit at 25° is 59.15 millivolts, as required by theory, may be as small as possible, the unknown solutions were referred to buffers of nearly the same acidity and the required pH derived by means of the equation

$$\mathrm{pH}_{\text{buffer}} - \frac{E_{\text{buffer}} - E_{\text{unknown}}}{0.05915} = \mathrm{pH}_{\text{unknown}} \qquad (6)$$

where E is the indicated potential in volts. No attempt was made to apply a correction for the liquid junction.

The pH values for the buffers were calculated on the basis of -0.3374_6 volt for the potential of the 0.1 M calomel electrode at 25°. The recent work of Randall and Young (7) and Roberts and Fenwick (1) leads to the conclusion that this potential is -0.3341 volt, making the pH assigned to the buffers 0.057 lower than their true values. The Clark and Lubs scale is the most generally accepted at the present time; therefore in our tables we have recorded our pH values on that basis in order to avoid confusion in case any attempt is made to check our measurements, but the

dissociation constants were computed from the true pH values. The working equations corresponding to Equations 4 and 5 are:

$$\log \gamma_{H^+} m - 0.5\, m^{\frac{1}{2}} + 2(\text{pH} + 0.057) = \log \frac{K'_b}{K_w} \qquad (4')$$

$$\log m + 2(\text{pH} + 0.057) = \log \frac{K''_b}{K_w} \qquad (5')$$

DISCUSSION.

Results on Local Anesthetics.

The data for the four new anesthetics are listed in Table I. Table IV gives a similar summary for procaine. The column headings are practically self-explanatory. Each value given for the measured pH represents an entirely independent experimental result; the determinations on duplicate solutions were sometimes made months apart. It will be observed both from these and from the values in the next column, which were read from the smoothed pH *versus* $m^{\frac{1}{2}}$ curve, that measurements made in dilute solution, 25 and 10 millimolal, are much more erratic than those made in higher concentrations. Such solutions are not good conductors; the potential readings are very hard to make and inclined to be inconsistent. For this reason these complex organic compounds, which at best are far more difficult to deal with than inorganic salts, are poor illustrations of the advantages of our method of obtaining dissociation constants by extrapolation because of the uncertainty of the length of the extrapolated portion of the curve.

The columns headed "$\log \frac{K'_b}{K_w}$" and "$\log \frac{K''_b}{K_w}$" contain the logarithms of the apparent dissociation constants at the stated concentrations, calculated by means of Equations 4′ and 5′ respectively. They demonstrate very clearly the inconsistency of the two sets of assumptions made in developing these equations and the impossibility of obtaining a trustworthy value for the dissociation constant from the measured acidity of a single dilution. The last column in Section A, Table I, has already been discussed.

The concentration range over which K'_b is equal to the true dissociation constant, K_b, is given by the limits within which $\frac{K'_b}{K_w}$ remains independent of the composition of the solution. This varies with the different anesthetics and is shortest with Sample E.P., which makes the extrapolation in this case the least certain. Fig. 4 shows the curves for the apparent dissociation constants of the new anesthetics and procaine; that for Sample E.P. is outstanding in shape because the pH *versus* $m^{\frac{1}{2}}$ curve for this substance is much steeper than that found for any of the others. As a result any slight error in judgment with regard to the region in which the slope of the latter curve becomes theoretical may affect the value of $\frac{K_b}{K_w}$ considerably. However, the order of decreasing basicity is very definite, beginning with Sample E.P. and ending with Sample E.E. in the order in which the table is arranged, procaine apparently falling between the first two members of the series.

The solubility of Sample B.P. is much the lowest of the group; measurements could not be made beyond 50 millimolal.

Samples βE.E. and E.E. behaved abnormally in the antimony electrode; instead of a continually increasing potential reading, decreasing values were obtained after the first few hours, indicating an apparent slow increase in acidity. These two compounds are the most acid of the series; the most probable explanation of their behavior seems to be slow ester hydrolysis. In order to show the effect of this hydrolysis on the apparent dissociation constant of the base, pH values were determined 2, 5, and 24 hours after the solutions under investigation were put in the antimony electrode and the apparent constants calculated for the three sets of data. Since the 2 hour value is usually not far from the crest of the time curve and allows a sufficient interval for the system to come to temperature and permit of a consistent comparison with the reference buffer to follow, the absence of any side reactions during the first 2 hours was assumed and the extrapolated value of $\frac{K'_b}{K_w}$ for the close of this period taken as the true constant $\frac{K_b}{K_w}$.

TABLE I.

Measured pH of Solutions of Several Local Anesthetics and Computed Dissociation Constants at 25°.

A. Hydrochloride of 2-ethoxy-3-diethylaminopropylnaphthoate (Sample E.P.).						B. Hydrochloride of 2-ethoxy-3-dibutylaminopropylnaphthoate (Sample B.P.).				
Concentration.	Measured pH.	Interpolated pH.	$\log \frac{K'_b}{K_w}$.	$\log \frac{K''_b}{K_w}$.	$\log \gamma_{H^+} m$.	Concentration.	Measured pH.	Interpolated pH.	$\log \frac{K'_b}{K_w}$.	$\log \frac{K''_b}{K_w}$.
mM per 1000 gm. H_2O						*mM per 1000 gm. H_2O*				
200	3.95	3.93_8	6.991	7.291	-0.775_7	50	4 57, 4 48	$4\ 57_1$	7.780	7.955
150	3.95	3.95_0	6.919	7.190	$-0\ 900_9$	40	4.60, 4.69	4.62_9	7.815	7.974
100	3.99	3.98_0	6.841	7 074	-1.074_7	25	4 67, 4.77	4.73_9	7.860	7.990
50	4.29	4.29_5	7.228	7.403	-1.364_5	20		4.78_9	7.875	7.993
25	4.86	4.85_9	8.100	8.230	-1.653_1	10	4.93, 5.01	4.94_2	7.912	7.998
20		5.03_5	8.367	8 485	-1.746_2	5		5.08_5	7.920	7.983
10	5.56	5.48_0	8.988	9.074	-2.035_7	2		5.27_3	7.920	7.961
5		5.81_3	9.376	9.439	-2.328_8	1		5.41_8	7.920	7.950
2		6.12_5	9.624	9.665	-2.718_1	0			7.920	
1		6.27_0	9.624	9.654	-3.014_5					
0			9.624							

Concentration.	2 hrs.				5 hrs.			24 hrs.		
	Measured pH.	Interpolated pH.	$\log \frac{K'_b}{K_w}$.	$\log \frac{K''_b}{K_w}$.	Measured pH.	Interpolated pH.	$\log \frac{K'_b}{K_w}$.	Measured pH.	Interpolated pH.	$\log \frac{K'_b}{K_w}$.
mM per 1000 gm. H_2O										
200	4.14, 4.03	4 06$_4$	7.243	7.543	3 95, 3 91	3.95$_2$	7.019	3.71, 3.75	3.73$_2$	6.579
150	4.14, 4.18	4.14$_6$	7 311	7.582	4 03, 4.10	4 03$_4$	7 087	3.81, 3.94	3.80$_6$	6.631
100	4.25, 4.22 4.21	4.24$_5$	7 371	7 604	4 08, 4.34	4.13$_7$	7.155	3.90, 4 09 3.74	3.89$_6$	6.673
50	4.50, 4.42 4.37	4.41$_5$	7.468	7.643	4 32, 4.13 4.10	4.31$_5$	7.268	4.20, 3.97 3.90	4.05$_0$	6.738
25	4.41, 4.78 4.32	4.57$_1$	7 524	7 654	4.30, 4 57 4 20	4.46$_8$	7.318	4.20, 4.50 4.01	4.21$_0$	6.802
20		4.61$_3$	7.523	7.641		4.51$_0$	7.317		4.26$_2$	6 821
10	4.48, 5.37	4.74$_8$	7.524	7.610	4.42, 5.17	4.64$_6$	7.318	4.35, 4.91	4.41$_7$	6.862
5		4.88$_7$	7.524	7.587		4.78$_4$	7.318		4.55$_6$	6.862
2		5.07$_5$	7.524	7.565		4.97$_2$	7.318		4.74$_4$	6.862
1		5.22$_0$	7.524	7.554		5.11$_7$	7.318		4.88$_9$	6.862
0			7.524				7.318			6.862
D. Hydrochloride of 2-ethoxy-3-diethylaminoethylnaphthoate (Sample E.E.).										
200	3.91, 3.77	3.87$_2$	6.859	7.159	3.84, 3.71	3 82$_4$	6.763	3.62, 3 50	3.59$_9$	6.313
150	4.08, 3.96	3 97$_8$	6.975	7.246	3 99, 3.90	3.91$_1$	6 841	3.76, 3.78	3.68$_2$	6.383
100	4.17, 4.08	4 11$_1$	7.103	7 336	4 05, 4 00	4 01$_7$	6.915	3.85, 3.73	3.79$_0$	6.461
50	4.40, 4.41	4.29$_7$	7.232	7.407	4.28, 4 32	4.19$_0$	7.018	4.05, 4 05	3.97$_2$	6.582
25	4.39, 4.51	4.45$_7$	7.296	7.426	4.31, 4 42	4.36$_4$	7.110	4.06, 4.13	4.15$_4$	6.690
20		4.49$_2$	7.281	7 399		4.42$_0$	7.137		4.21$_2$	6.721
10	4.64, 4.63	4.63$_5$	7.298	7.384	4 58, 4.58	4 58$_5$	7.198	4.54, 4.31	4.37$_1$	6.770
5		4 77$_4$	7 298	7.361		4.72$_4$	7.198		4.51$_0$	6.770
2		4.96$_2$	7.298	7.339		4.91$_2$	7.198		4.69$_3$	6.770
1		5.10$_7$	7.298	7.328		5.05$_7$	7.198		4.84$_3$	6.770
0			7.298				7.198			6.770

The solutions of Samples βE.E., E.E. and, to a lesser degree, some others included in the work, frequently seemed to have a

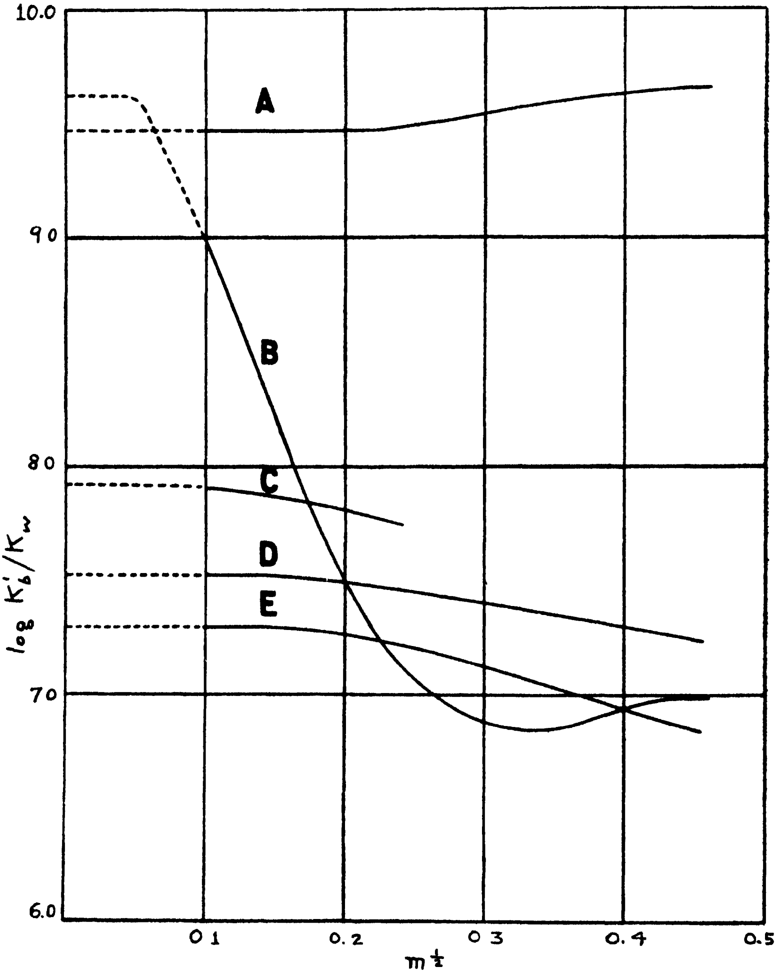


Fig. 4. The apparent dissociation constant of some local anesthetics at various concentrations. Curve A, procaine; Curve B, Sample E.P.; Curve C, Sample B.P.; Curve D, Sample βE.E.; Curve E, Sample E.E.

permanent effect upon the potential of the measuring electrode. Buffer solutions run between each concentration of anesthetic did

not agree among themselves but tended to give lower potentials with successive runs unless alkali pretreatment of the electrode was resorted to. The total absence in the electrode, after many months of use, of any solid phase other than the original cubic antimony trioxide precluded an explanation for this phenomenon based on the instability of cubic antimony trioxide in the presence of the compounds studied. We have no explanation to offer which seems to us sufficiently general; yet the consistency of the results obtained by comparing each solution investigated with a buffer measured immediately after led to the conclusion that the buffer irregularity was not of major importance from the standpoint of the degree of accuracy required for the determination of pH.

Dissociation Constant of Diethylaminoethanol at 25°.

Because of the unusual behavior of Samples βE.E. and E.E. the hydrochloride of the alcohol common to both preparations, diethylaminoethanol, was studied; Table II contains the results. No difficulties or abnormalities of any kind were encountered. The dissociation constant is considerably higher than that for either of the anesthetics, as is to be expected.

In this case the free base itself is soluble in water; therefore, as a check on our method of calculating the dissociation constant of a base from pH measurements made on its hydrochloride, the acidity of several concentrations of the base was measured, although with great difficulty, and compared with the values for the same solutions calculated from the constant obtained in Table II. (See Table III.) The alcohol is so slightly ionized that the readings are very hard to make and uncertain. The computed acidities were derived from the approximation equation $2\ (\mathrm{pH} + 0.057) = \log \frac{K_b}{K_w} - \log K_w + \log m$; the values assigned the two constant terms were: $\log \frac{K_b}{K_w} = 8.770$ and $\log K_w = -14$. The equation follows directly from the expression for the ionization constant of a base, on the assumption that the activity coefficients of both ions are the same and the activity of the undissociated fraction equal to the total quantity of base present.

These assumptions are undoubtedly not free from error, yet they lead to calculated values which agree within the experimental accuracy with those determined in the presence of air. Determinations were made in both air and nitrogen, as was also the case with procaine, with a slightly modified form of our regular

TABLE II.

Measured pH of Solutions of the Hydrochloride of Diethylaminoethanol and Computed Dissociation Constant of the Base at 25°.

Concentration.	Measured pH.	Interpolated pH	$\log \frac{K'_b}{K_w}$.	$\log \frac{K''_b}{K_w}$.
mM per 1000 gm. H_2O				
200	4 71, 4 74	4 72_8	8 571	8.871
150	4 83	4 79_4	8.607	8.878
100	4 85, 4 89	4 88_5	8.651	8.884
50	5 07, 5 05	5 04_2	8.722	8.897
25	5 17, 5 45	5 18_9	8.760	8.890
20		5 23_6	8.769	8.887
10	5 03, 5.54	5.37_1	8.770	8.854
5		5 51_0	8 770	8.833
2		5 69_8	8 770	8.811
1		5 84_3	8 770	8.800
0			8 770	

TABLE III.

Measured and Computed pH of Solutions of Diethylaminoethanol at 25°.

Concentration	Measured pH (air).	Measured pH (N_2).	Computed pH.
mM per 1000 gm. H_2O			
200	10.91	11 30	10.98
150	11 01	11 28	10 92
100	10 92	11 17	10.83

antimony trioxide electrode for the nitrogen work (1). In the case of diethylaminoethanol the two sets do not agree well, probably due to the experimental difficulty attending any attempt to make potential measurements on this substance since no such significant differences were found with procaine.

Relation of the Dissociation Constants of Local Anesthetics to Their Anesthetic Efficiency.

The order of the dissociation constants for the local anesthetics, including procaine, has been mentioned. These same compounds were subjected to test in the Pharmacological Laboratory of Yale University, the detailed results of which will form part of a paper now in preparation by Professor Underhill. Sample E.P. was found to be more potent than procaine, possessing greater penetrating power and producing longer continued anesthesia. Sample B.P. came second in the naphthalene series in decreasing order but was found considerably less effective than procaine. Samples βE.E. and E.E. were much less pronounced in effect, both in respect to potency and duration, than the first two members of the series, Sample βE.E. appearing slightly the better. It will be observed from this that the order of decreasing anesthetic efficiency is the same as the order of the decreasing strength of the free bases. In line with this generalization is the fact that Sample E.P. was found relatively more effective with increasing dilution. As has been pointed out, solutions of this material decrease in acidity more rapidly on dilution than do those of any of the related compounds.

Relation of the Dissociation Constants of Local Anesthetics to Their Chemical Structure.

As the result of a number of reports in the literature correlating the efficiency of local anesthetics with the acidity of their solutions, Vliet and Adams (8) were led to determine the dissociation constants of a considerable number of compounds structurally related to procaine, using a hydrogen electrode. They found the basicity to decrease as the size of the groups attached only to the nitrogen increased, whereas an increase in the number of carbon atoms in a straight chain between the nitrogen and oxygen atoms had the opposite effect. Our own results with Samples E.P., B.P., and E.E. are in complete harmony with this generalization; Sample βE.E. does not permit of comparison because of the absence of the ethoxy group.

Dissociation Constant of Procaine at 25°.

From Table IV it is apparent that for procaine at 25°, $\log \frac{K_b}{K_w} = 9.476$, whence $K_b = 29.9 \times 10^{-6}$. Kolthoff (9), employing color indicators, obtained the value 7.1×10^{-6}; Vliet and Adams (8) give 1250×10^{-6}, derived from the measured pH of 6.4 for a 0.02 N solution. The discrepancy between the Kolthoff constant and our own is not surprisingly large in view of the difference in method and calculation, but the lack of any sort of agreement with

TABLE IV.

Measured pH of Solutions of Procaine and Computed Dissociation Constant at 25°.

Concentration.	Measured pH (air).	Measured pH (N_2).	Interpolated pH.	$\log \frac{K'_b}{K_w}$.	$\log \frac{K''_b}{K_w}$.
mM per 1000 gm. H_2O					
200	5 33	5.22	5.26_8	9.651	9.951
150		5.24	5.30_1	9.621	9.892
100	5.41	5.34	5.33_8	9.556	9.790
50	5.47	5.40	5.42_2	9.482	9.657
25	5.58	5.56	$5\ 54_7$	9.476	9.604
20			5.59_0	9.477	9.595
10	5.50	6.63	5.72_4	9.476	9.562
5			5.86_3	9.476	9.539
2			6.05_1	9.476	9.517
1			6.19_6	9.476	9.506
0				9 476	

that of Vliet and Adams is disconcerting. The pH in Table IV corresponding to theirs is 5.59_0, about 0.8 pH more acid. It seemed essential, therefore, to compare the Vliet and Adams results with hydrogen electrode measurements of our own in order to justify the use of the antimony electrode for similar determinations. Prior to this another substance, aniline hydrochloride, the dissociation constant of the base of which has been determined by a number of other investigators, was selected for test with the antimony electrode.

Dissociation Constant of Aniline at 25°.

From Table V it appears that the dissociation constant of aniline at 25° is 2.13×10^{-10}. Denham (10) and Loomis and Acree (11), also employing an electrometric method, found 4.62×10^{-10} and 4.10×10^{-10}, respectively, for the constant. Tizard (12), with the aid of color indicators, obtained 4.13×10^{-10}, Lunden's (13) hydrolysis method yielded 4.6×10^{-10}, while Bredig (14) found 4.17×10^{-10} conductimetrically. It is very difficult to make any sort of reasonable comparison of our data with that of others.

TABLE V.

Measured pH of Solutions of Aniline Hydrochloride and Computed Dissociation Constant of Aniline at 25°.

Concentration	Measured pH.	Interpolated pH	$\log \frac{K'_b}{K_w}$.	$\log \frac{K''_b}{K_w}$.
mM per 1000 gm. H_2O				
200	2 67	2 60_7	4 329	4 629
150	2 71	2 65_5	4 329	4 600
100	2 75, 2 71	2 72_4	4 329	4 562
50	2 77	2 84_5	4 328	4 505
25	2 98	2 97_3	4 328	4 460
20		3 01_6	4 329	4 447
10	2 98	3 15_0	4 328	4 414
5		3 28_9	4 328	4 391
2		3 47_7	4 328	4 369
1		3 62_2	4 328	4 358
0			4 328	

The assumptions involved in the older work are quite radically different, the experimental methods were cruder than those now employed, and the adopted reference values inconsistent. An attempt to recalculate the data seemed hopeless because so many of the difficulties were inherent in the data. Perhaps the best comparison obtainable is to take the mean of the constants cited, 4.3×10^{-10}, which, calculated by Equation 4′ gives 3.30 pH at 10 millimolal. The agreement with our interpolated value (3.15) is not good, but, judged from the measured result is not impossible in magnitude.

TABLE VI.

Comparison of Results Obtained with Hydrogen and Antimony Electrodes at 25°.

Sample.	Concentration.	Computed pH.	Measured pH.		Comments on behavior of hydrogen electrode.
			Antimony.	Hydrogen.	
	mM per 1000 gm. H_2O				
E.P.	100		3.98	4.63	Good reading, held from 3rd to 24th hr.
B.P.	40		4.63	4.64	Reading fairly constant at 24 hrs.
βE.E.	100		4.25	4.62–4.37	Steady fall from 15 min. to 24 hrs.
Diethylaminoethanol hydrochloride.	100		4.89	4.77–5.35	Steady rise 2 to 30 hrs.
Diethylaminoethanol.	100		(Air.) 10 92 (N_2) 11.17	11.23	Difficult to read, error 2 mv.
Procaine.	100		5.34	5.64	Constant value 3rd to 6th hr.
	100		5.34	5.28–5.81	Continued rise over 24 hrs.; no constant value.
	25		5.55	5.69–5.79	Rose 0.1 pH between 3rd and 20th hr.
Aniline hydrochloride.	100	2.88	2.72	2.53–2.62	Fluctuated with no specific trend; 2 mv. fluctuations about mean.
	50	2.94	2.85	2.76	
Benzoic acid.	25	2.84	2.95	2.76	Rise of about 4 mv., 2nd to 24th hr.
p-Aminobenzoic acid.	25	3.60	3.63	3.79	Difficult to read, error 2 mv.; held 10 hrs.

Comparison of Results Obtained with Hydrogen and Antimony Electrodes.

In order to pursue further the subject of the discrepancy between the results obtained with the antimony electrode and those by other methods, with particular regard to the great disagreement observed with procaine, a hydrogen electrode was set up and a number of solutions measured. Table VI is an outline of the results. At first glance the outstanding thing seems to be an almost complete lack of agreement between the two methods, or, indeed, between two sets of results obtained by the same method by different workers, since the recorded hydrogen electrode values for procaine are far too low to be comparable with that of Vliet and Adams. There are, however, some general conclusions evident, chief among which is the fact that the potential of the hydrogen electrode varied widely with time. It has been customary with other workers to accept arbitrarily a reading that changed little during not longer than the course of the 1st hour, sometimes over a much shorter period. Our results show that such a value is by no means trustworthy, equilibrium being much delayed in some cases, or apparently never actually attained, the trend with time always being toward increased alkalinity except in the case of Sample βE.E., when the change was in the opposite direction, thus checking our findings with the antimony electrode as regards the apparent hydrolysis of this compound.

Unpublished and scattered data in this and other laboratories have indicated that in the case of benzoic acid the constant obtained electrometrically is in discord with that from other methods, the acid appearing much too strong. Acting on the belief that this effect may be generally ascribed to selective absorption by the platinum black coating of the electrode, we used a very thin plate in our experiments. The result is in much better agreement than those obtained with a heavy plate although still numerically the smallest of the three values cited.

The column headed "Computed pH" contains in the case of aniline hydrochloride the values calculated from the mean dissociation constant discussed in the last section. Conductivity data on benzoic acid by White and Jones (15) and Schaller (16) yield an average value for K_a of 6.79×10^{-5}. With the approxi-

mation equation $\log K_a = -2(\mathrm{pH} + 0.057) - \log m$, the recorded pH was derived. Walker (17) gives $K_a = 1.21 \times 10^{-5}$ and $\frac{K_b}{K_w} = 210$ for *p*-aminobenzoic acid. With the ordinary equation for the acidity of amphoteric electrolytes, $-2(\mathrm{pH} + 0.057) = \log (K_a m + K_w) - \log \left(\frac{K_b}{K_w} m + 1\right)$, a pH of 3.60 was calculated for a 25 millimolal solution. In the main the computed acidities check the antimony results the more closely. It may be mentioned once more that any comparisons made with old data are of questionable significance because of the lack of consistency with recent theories, but it seems at least fairly evident that the results given by the hydrogen electrode on complex organic compounds of the type taken up in this paper are extremely doubtful, as has occasionally been hinted in the literature, and their accuracy in greater suspicion than those of the antimony electrode.

CONCLUSION.

The purpose of this paper is not so much to present data for a series of rather unusual compounds which, as a group, may never be of outstanding interest, but to attract attention to an apparently fertile field of research and to point out the probable inaccuracies in the conclusions that have been drawn from pH determinations made on complex organic compounds. That there is a definite correlation between the efficiency of local anesthetics of a *related series*, their basic dissociation constants, and their chemical structure seems positive; it is possible that any local anesthetic to be effective must have a basic constant within a certain range of values. A determination of the constant should, therefore, prove a valuable aid in predicting the anesthetic potency of a given compound and a guide to further synthesis.

Heretofore it has been usual to calculate dissociation constants from the measured pH of a single solution, or at most a very few solutions of the substance under investigation. We have attempted to demonstrate that reliable values for such constants can be obtained only by the extrapolation of a considerable number of determinations covering a fairly wide range in concentra-

tion to infinite dilution, since it is only at infinite dilution that the assumptions involved in the computations are valid.

The usefulness of the antimony-antimony trioxide electrode has been emphasized, particularly with regard to its probable increased accuracy over the hydrogen electrode in work of this kind. Certain types of organic compounds are extremely difficult to measure with the hydrogen electrode, due possibly to selective absorption by the platinum black coating of the electrode or to the catalysis by it of side reactions.

SUMMARY.

1. Acidity determinations have been made at 25° with the antimony-antimony trioxide electrode on a series of solutions of four new local anesthetics of the naphthalene series, together with procaine, diethylaminoethanol, and aniline hydrochloride.

2. From these sets of values the corresponding basic dissociation constant was computed by an extrapolation involving the Debye-Hückel equation for the activity coefficient of an ion at vanishing concentration. This constant was compared in each case with the apparent dissociation constants calculated for the several dilutions measured.

3. The order of increasing basicity of the four related anesthetics proved to be the same as the order of increasing anesthetic efficiency.

4. The relationship between the order of increasing basicity and the chemical structure of the members of the naphthalene series was found to be the same as that discovered for the procaine series by Vliet and Adams.

5. Comparison measurements made with the antimony and hydrogen electrodes on solutions of the compounds named and also benzoic acid and *p*-aminobenzoic acid failed to agree; the behavior of the hydrogen electrode is shown to be erratic and untrustworthy.

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THE EFFECT OF INSULIN UPON THE BLOOD AMINO ACID NITROGEN OF THE RABBIT.

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INTRODUCTION.

Luck *et al.* (1) and Kerr and Krikorian (2) recently reported that a hypoaminoacidemia followed the injection of insulin in man, the rat, the rabbit, and the dog. The original purpose of the present paper was to determine whether the denatured insulin of Bischoff (3), which was no longer acid-soluble and in some cases was biuret-negative, was able to affect the blood amino acid nitrogen.[1]

In testing out this question samples of iletin (the insulin of Eli Lilly and Company), crystalline insulin, and a composite of our denatured insulins were standardized in terms of rabbit units by our method of insulin assay (4). A series of these same standard rabbits was then given maximum subconvulsive doses of the three insulins, the blood sugar and amino acid nitrogen values being determined. The results were entirely unsatisfactory, neither of the three insulins having any appreciable effect upon the amino acid nitrogen. In this series of experiments there were two

[1] If the denatured insulin produced no hypoaminoacidemia, or if the degree of hypoaminoacidemia was equal to that of the starting material, it would be established that the insulin protein contained at least two active chemical groups, one concerned with hypoglycemia, and the other with the amino acid metabolism. If the effect upon the amino acid nitrogen of the blood was diminished to the same degree that the hypoglycemic properties were diminished by denaturation, the evidence would point to a "single active group." Such a series of experiments could of course not settle whether this active group functioned directly to bring about deaminization or whether the disappearance of the amino acids was stimulated in some way by the hypoglycemia or a change in the glycogen reserves.

points of departure from Luck's series, *viz.*, the animals were fasted 24 instead of 48 hours, and "standard" rabbits, which had had convulsions weekly for a long period of time, were used. Rabbits fasted 48 hours, and rabbits which had never had insulin before were then tried. The results were essentially negative, the fall in amino acid nitrogen following the insulin being of scarcely greater magnitude than the fall in several of the controls.

Purpose.

In this paper an attempt was made to establish the conditions under which an insulin hypoglycemia in the rabbit was accompanied by a definite hypoaminoacidemia. Blood sugar, amino acid nitrogen, and, in some cases, urea nitrogen were determined at intervals under the following conditions: (1) control rabbits fasted 24 and 48 hours; (2) rabbits fasted 24 and 48 hours, and given a single subconvulsive dose of insulin; (3) rabbits fasted 24 hours, and given a series of doses of insulin, so that a prolonged hypoglycemia resulted; (4) rabbits fasted 24 hours, and subjected to an amino acid tolerance test; (5) fasted rabbits first given adrenalin to mobilize the glycogen stores, and then given insulin 24 hours later, in single and repeated doses.

EXPERIMENTAL.

Procedure.

The rabbits were bled from the marginal ear vein, 3 cc. of blood being taken for each analysis. Since seven or more bleedings were made in the course of some experiments approximately 10 per cent of the total blood was taken in these experiments.

The methods of Folin and Wu were used for the determination of sugar, amino acid nitrogen, and urea nitrogen. To be certain that the amino acid nitrogen method in our hands was not giving erratic results, at the time these determinations were made, simultaneous determinations of known amounts of glycine added to blood filtrates were made. Several series of the amino acid nitrogen values reported were run in duplicate.

Control Rabbits.

In Table I are recorded the amino acid blood nitrogen values of two series of rabbits, one which had been fasted 24 hours, the other 48 hours. In the rabbits fasted 48 hours, there is a definite falling off of the value, after successive bleedings, an observation apparent in only one of the rabbits fasted 24 hours. The rabbits

TABLE I.

Control Rabbits. Effect of Fasting and Bleeding upon Blood Amino Acid Nitrogen.

Time fasted.	Rabbit No.	Amino acid N, mg. per 100 cc.						Maximum increase.	Maximum decrease.
		Before bleeding.	After bleeding.						
			1½ hrs.	3 hrs.	5 hrs.	7 hrs.	24 hrs.		
hrs.								*per cent*	*per cent*
24	5	14.9	13.3	14.0	12.2	12.9	15.4	3	18
	1	11.1	13.3	13.7	11.8	12.5	15.4	39	0
	2	10.0	10.9	11.4	10.4	9.0	9.8	14	10
	3	11.1	10.6	11.1	12.5	10.0	10.3	13	11
	123	9.0	10.7	10.9	9.8	9.3	11.2	24	0
	131	10.0	10.3	11.2	10.0	8.7	7.7	11	23
	C	11.2		10.7		10.6		0	6
	W	8.5		8.3		8.7		2	2
Average								13	9
48	2′	12.3	11.7	9.0	9.3			0	27
	4	11.0	10.3	10.4	11.0			0	5
	3′	11.8	10.4	10.5	10.8			0	12
	1′	12.1	9.8	9.4	10.3			0	22
Average								0	16.5

fasted 24 hours, show a considerable variation in both directions. An examination of the amino acid nitrogen data in a paper from this laboratory showed a similar fluctuation. (See experiments with guanylpiperazine, isoamylguanidine, creatinine, creatine (5).)

Effect of a Single Subconvulsive Dose of Insulin.

"Standard" rabbits and rabbits which had not before received insulin were used. In this series of experiments an attempt was

TABLE II.

Effect of Single Dose of Insulin upon Blood Amino Acid Nitrogen of Rabbits.

Time fasted.	Rabbit No.	Date.	Insulin.	Amino acid N, mg. per 100 cc. Before insulin.	After insulin. 1½ hrs.	3 hrs.	5 hrs.	Maximum increase.	Maximum decrease.
hrs.		*Nov.*	*units per kg.*					*per cent*	*per cent*
24	123	13	3.0 Denatured.	9.6	8.9	8.5	7.8	0	19
	122	13	2.5 "	8.9	8.7	10.3	9.1	16	0
	78	5	0.6 "	12.4	7.9	7.9	8.5	0	36
	78	13	0.6 "	10.0	10.0	8.3	8.5	0	17
	12	5	0.7 "	10.0	9.1	8.5	9.1*	0	15
	12	13	0.6 "	9.9	8.0	8.3	9.1	0	19
Average								3	17
48	123	20	3.0 Denatured.	9.1	8.5	9.1	8.5	0	7
	122	20	2.5 "	10.0	7.7	9.8	7.6*	0	24
	1"	21	5.2 "	10.0	8.5	8.7	6.9	0	31
	2	21	5.2 "	9.3	7.7	8.4	7.2	0	23
Average								0	21
24	33	5	0.8 Iletin.	8.6	8.5	9.7	8.9	13	0
	33	18	0.8 "	10.3	10.3	8.7	9.5	0	16
	26	5	1.7 "	11.8	8.7	10.0	10.5	0	26
	26	13	1.7 "	8.6	9.0	9.5	8.3	10	3
Average								4	11
48	3	20	5.0 Iletin.	9.4	9.4	9.1	8.2	0	13
	5	20	5.0 "	9.4	9.3	9.5	6.7	0	29
Average								0	21
24	29	5	0.95 Crystalline.	12.4	11.3	10.5*	11.8	0	16
	29	13	0.85 "	10.5	10.5	10.0	10.5	0	5
	1	5	1.0 "	9.8	9.5	9.5	9.5	0	3
	1	13	1.0 "	9.0	8.3	9.3	8.2	3	8
Average								1	8
48	6	20	5.0 Crystalline.	12.1	9.8	9.4	10.3	0	22

* Convulsions.

made to produce a maximum hypoglycemia without convulsions by a single dose of insulin. Of the twenty-one rabbits recorded, a 50 per cent or greater drop in blood sugar was produced in all but two of the rabbits, for which it was 40 per cent. Three of the rabbits had convulsions. The blood sugar values have not been included, because they are irrelative to an interpretation of the results. The data are assembled in Table II. The rabbits which had fasted 48 hours showed a greater decrease in amino acid nitrogen than those fasted 24 hours. The drop is only of slightly greater magnitude than that of the controls, however. The average drop of the seven rabbits, fasted 48 hours, is 22 per cent; the four controls averaged 16.5 per cent. The maximum drop is 31 per cent for the dosed rabbits and 27 per cent for the controls. The minimum drop is 7 per cent for a dosed rabbit and 5 per cent for a control. No difference in effect between denatured insulin, iletin, or crystalline insulin can be detected. The rabbits which had fasted 24 hours do not show as great a fluctuation of values above and below the initial value as do the controls. The average decrease for the rabbits, which had received iletin and crystalline insulin, is the same as for the controls; *viz.*, 8 and 11 per cent, against 9 per cent. The values for the denatured insulin average 17 per cent. The maximum decrease for the dosed rabbits is 36 per cent; the maximum for the controls is 23 per cent. In several instances the experiment was repeated 1 or 2 weeks later upon the same rabbit. Thus Rabbit 78 showed a maximum drop of 36 per cent in amino acid nitrogen 1 week and 17 per cent the following week. Rabbit 33 showed no drop and a rise of 13 per cent 1 week and no rise and a drop of 16 per cent the following week. The results for other rabbits, Nos. 12, 122, 29, 26, and 1, are included in Table II.

Effect of a Prolonged or Repeated Hypoglycemia.

The effect of repeated doses of insulin upon the urea and amino acid nitrogen was followed in a rabbit, which was kept in hypoglycemia for 10 hours. The data are given in Table III, and are the only indications of a definite hypoaminoacidemia obtained in all of our experiments. The animal had been fasted 24 hours before the first dose of insulin was given. Two other rabbits were

each given two subconvulsive doses of insulin 24 and 48 hours after fasting. There is no indication of a hypoaminoacidemia in these two rabbits.

Amino Acid Tolerance.

In his study on the effect of synthalin on metabolism (6) Blatherwick included data for one experiment in which insulin was

TABLE III.

Effect on Blood of Prolonged or Repeated Hypoglycemia.

Results are expressed in mg. per 100 cc. of blood.

Time.	Rabbit 6.				Rabbit 2.				Rabbit 3.			
	Insulin.	Sugar.	Urea N.	Amino acid N	Insulin.	Sugar.	Urea N.	Amino acid N.	Insulin.	Sugar.	Urea N.	Amino acid N.
hrs.	*units*				*units*				*units*			
Before insulin.	15	97	14 7	12.5	15	103	26.2	11.0	15	85	29.5	12.3
After insulin.												
2		80	14.7	11.1								
3	15											
4		58	13.7	10.9		78	24.8	8.7				10.0
6	7	67	15.7	9.5								
8		36*	11.6*	8.7*		96	35.8	11.6		85	30.3	12.5
9	7											
12		28	17.7	7.7								
24	Death between				15	105	24.5	9.8	21	82	29.5	10.1
26	15th and 24th				5	74	31.3	10.0		30	29.5	10.0
29	hrs.					78	27.0	9.1		46	33.0	9.0
31						93	33.0	9.1		73	26.0	11.1
48						90	33.0	11.4		87	26.0	10.2

* Blood clotted.

given simultaneously with a glycine tolerance test. The curve was similar to that for Rabbit A which had received no insulin. Blatherwick's four control rabbits of the glycine tolerance showed an appreciable variation in the different animals. We produced in two rabbits a glycine tolerance and repeated the tolerance along with a hypoglycemic dose of insulin several weeks later upon the same rabbits. The data are given in Table IV. No conclusions can be drawn.

TABLE IV.

Amino Acid Tolerance.

Glycine, 1.75 gm. per kilo, was given subcutaneously in each experiment.

Rabbit No.	Date.	Amount in 100 cc. blood.							Remarks.
		Substance.	Before glycine.	After glycine.					
				1½ hrs.	3 hrs.	5 hrs.	7 hrs.	24 hrs.	
131	Jan. 31	Sugar.	95	111	95	95	95	95	Control.
		Urea N.	29.5	44.5	33	32.3	41.0	29 5	
		Amino acid N.	12.1	24.2	22 2	24.2	20.0	10.8	
	Feb. 11	Sugar.	106	61	56	56	32	129	Two doses of insulin, 1st and 4th hrs.
		Urea N.	24.5	25.2	37.0	30.3	25.2	31.3	
		Amino acid N.	10.3	23.6	23 6	20.0	23.6	10.6	
128	Jan. 31	Sugar.	100	118	125	129	133	118	Control.
		Urea N.	31.5	32.5	36 3	45.5	52.1	34.8	
		Amino acid N.	10.8	24.2	24 8	23.6	21.6	11.8	
	Feb. 11	Sugar.	112	63	67	63	33	112	Two doses of insulin, 1st and 4th hrs.
		Urea N.	25.0	27.8	35.1	30.3	27.3	26.0	
		Amino acid N.	10.0	21.6	27.6	20.0	15.6	10.3	

TABLE V.

Premobilization of Glycogen Followed by Insulin Hypoglycemia.

Results are expressed in mg. per 100 cc. of blood.

Time.	Rabbit 5.				Rabbit 1.				Rabbit 4.			
	Insulin.	Blood sugar.	Blood urea N.	Blood amino acid N.	Insulin.	Blood sugar.	Blood urea N.	Blood amino acid N.	Insulin.	Blood sugar.	Blood urea N.	Blood amino acid N.
hrs	*units*				*units*				*units*			
Before insulin.	14	100	19.4	11.4	29.0	65	24 8	11.8	35	68	21.8	12.4
After insulin.												
2		67	19.4	11.6		24	26.0	10.0		26	23.5	10.5
4	15*	29	18.3	10.8		28	31.3	9.8	*	32	18.5	9.1
6	*	24	25 0	10 0		26	32 3	8.2		29	20.8	8.6
8		24	33.0	13.4†		49	34.5	9 3	*	33	20.5	9.4
12		55	31.0	10 9								
15	7											
24		63	35 3	9 3		98	29 5	10 5		83	24.8	9.5

* Convulsions.

† Blood was viscous.

TABLE VI.

Effect of Adrenalin upon Blood Amino Acid Nitrogen.

Results are expressed in mg. per 100 cc. of blood.

Time.	Rabbit 104.			Rabbit 57.			Rabbit 1.			Rabbit 4.		
	Sugar.	Urea N.	Amino acid N.	Sugar.	Urea N.	Amino acid N.	Sugar.	Urea N.	Amino acid N.	Sugar.	Urea N.	Amino acid N.
hrs.												
Before adrenalin.	105	28.5	10.0	114		9.8	83	24.0	11.1	82	22.8	10.8
After adrenalin.												
1½	258	27.0	8.7	228	27.8	8 7						
4	328	27.0	8.7	276	26.2	8 2						
6	276	30.2	8.3	200	32.2	9.5						
24	93	26.2	8 9	93	31.2	8.9	68	29.5	11.8	65	24.8	9.5

Premobilization of Glycogen by Adrenalin.

Cori and Cori (7) showed for rats, and Blatherwick and Sahyun (8), in this laboratory, for rabbits, that adrenalin raises the glycogen content of the liver. The carbohydrate supplies outside of the liver must in some way be depleted to furnish the increased liver glycogen. It is possible that this change in glycogen distribution might affect the action of insulin upon the amino acid nitrogen metabolism. Three rabbits were fasted 12 hours, and given each 1 mg. of adrenalin. 24 hours later insulin was given, in repeated doses for one rabbit and in single massive doses for the two other rabbits. The results are given in Table V. The fall in amino acid nitrogen is no greater than was observed in control rabbits, fasted for the same period. It is of interest to note that the blood sugar values 24 hours after the administration of adrenalin were low in Rabbits 1 and 4. The blood sugar values at the beginning of the experiment were 83 and 82. The effect of 1 mg. of adrenalin upon the blood sugar, urea, and amino acid nitrogen was followed for several rabbits. The results are given in Table VI. It will be noted that the amino acid nitrogen varies much the same as it does for the controls, there being a slight falling off from the initial value.

SUMMARY.

The amino acid nitrogen of the blood of fasting rabbits shows considerable variation when a series of bleedings is made during 24 hours. The value has a tendency to fall when the animals have fasted 48 hours.

Of twenty-five rabbits, given subconvulsive doses of insulin, only one showed a drop in amino acid nitrogen sufficient to indicate a hypoaminoacidemia. It was necessary to keep this animal in a prolonged state of hypoglycemia by repeated doses of insulin to show the effect.

No effect of adrenalin upon the blood amino acid nitrogen of the rabbit was noted. Premobilization of glycogen followed by insulin hypoglycemia was also without action.

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A CRYSTALLINE ALDOBIONIC ACID DERIVED FROM GUM ARABIC.*

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In a recent communication (1) it was shown that by partial acid hydrolysis of gum arabic in the cold an immunologically specific carbohydrate could be isolated, comparable in its precipitating activity for Types II and III antipneumococcus sera with the bacterial "soluble specific substances" themselves. On further hydrolysis the carbohydrate yielded galactose and two or more complex sugar acids, one of which appeared to be a disaccharide (aldobionic) acid similar to those isolated from the specific polysaccharides of the Type III pneumococcus and the Type A Friedländer bacillus.

Cretcher and Butler (2) also obtained indications of the presence of an aldobionic acid among the hydrolysis products of gum arabic itself, and have since (3) confirmed their preliminary findings, isolating the aldobionic acid through the calcium salt and demonstrating its structure to be that of a glucuronogalactose, although they refer to it in their paper as galactosoglucuronic acid as well.

As indicated in a footnote in our paper ((1), p. 854) the aldobionic acid has now been obtained in crystalline form through its crystalline cinchonidine salt. As the result of improved methods of separation, which will be described below, and possibly also through the infection of the laboratory with the original crystals, it was then found possible to obtain the crystalline acid without recourse to the cinchonidine salt.

Like the product isolated by Butler and Cretcher, the crystalline aldobionic acid possesses the structure of a glucuronogalactose;

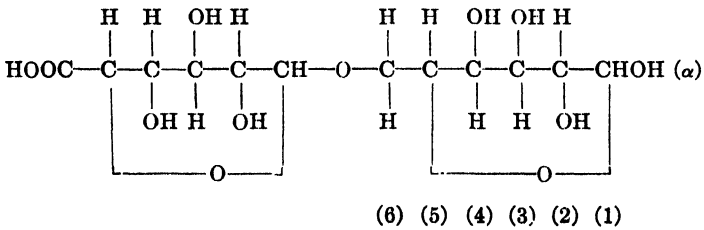
* The work reported in this paper was made possible by the Harkness Research Fund of the Presbyterian Hospital.

that is, a disaccharide composed of galactose and glucuronic acid, linked through the aldehyde group of the glucuronic acid. It will be recalled that a similar structure was indicated for the aldobionic acids derived from Type III pneumococcus (4) and Type A Friedländer bacillus (5), except that the second component was found to be glucose and not galactose.

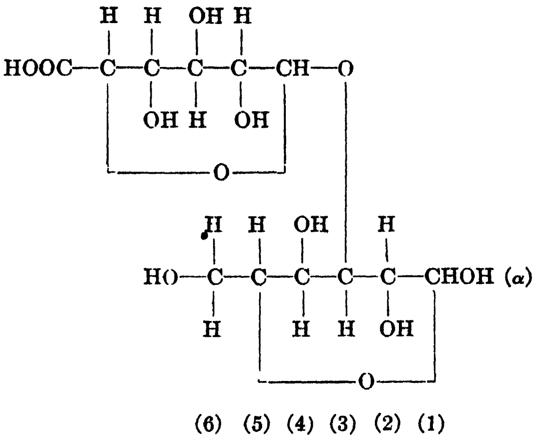
Unlike Butler and Cretcher's product, the crystalline acid shows no tendency to lactonize. This discrepancy is apparently due to the presence in the whole gum of a lactone-forming acid which is removed, together with arabinose, when the specific gum is formed by partial hydrolysis in the cold. While Butler and Cretcher report that their sample of arabic acid existed in the lactone form to the extent of about 22 per cent, only traces of lactone-forming material occur in the specific gum. These were recovered as a small dicarboxyaldotetrasaccharide fraction in the mother liquors from about 600 gm. of specific gum, as described below. It would therefore appear that Butler and Cretcher's aldobionic acid contains appreciable amounts of a second acid, probably also aldobionic in structure.

The crystalline aldobionic acid shows marked mutarotation in aqueous solution, the specific rotation changing from an initial reading of about + 12° to an equilibrium value of −8.5°, most of the change taking place within the 1st hour. The crystals would therefore appear to be the α form of the disaccharide.

Although no direct evidence is presented as to the point of attachment of the glucuronic acid portion of the aldobionic acid to the galactose, it is believed that the experiments below eliminate all but carbon atoms 3 and 6, and the formula of the crystalline aldobionic acid may provisionally be written as:



or



These formulas are based on the following experimental evidence.

1. Titration of the acid and analysis of the cinchonidine salt show one carboxyl group to be free in the molecule.

2. One reducing group is present and reacts as an aldehyde.

3. Hydrolysis of the aldobionic acid is very slow. Therefore the sugar in glucosidic union is probably not present in the γ or $<1, 4>$ oxidic form.

4. From the products of hydrolysis galactose has now been isolated in crystalline form and further identified as its osazone. The acid portion of the aldobionic acid is shown to be glucuronic acid by isolation of crystalline cinchonine glucuronate and glucuronic acid *p*-bromophenylosazone, and by the further fact that oxidative hydrolysis yields saccharic as well as mucic acid.

5. On oxidation the crystalline aldobionic acid yields a dicarboxybionic acid which forms a crystalline calcium salt. This still gives the naphthoresorcinol test for -uronic acids, and on hydrolysis yields crystalline cadmium galactonate, oxidizable to mucic acid, and glucuronic acid, identified through the *p*-bromophenylosazone and by its behavior on oxidation. Glucuronic acid is thus shown to be the portion of the aldobionic acid in glucosidic union, and galactose the portion containing the free aldehyde group. The crystalline acid is therefore glucuronogalactose, a conclusion already arrived at by Butler and Cretcher for

the amorphous acid from whole gum arabic. Furthermore, the crystalline acid is the α form of the aldobionic acid, since the rotation rapidly decreases on solution in water.

6. Since the aldobionic acid is a reducing sugar, the glucuronic acid cannot be attached to carbon atom 1 of the galactose.

7. The aldobionic acid forms osazones, and although no crystalline products could be obtained, the indication of their formation is believed to eliminate carbon atom 2 as the point of attachment.

8. Carbon atom 4 of the galactose is eliminated since the dicarboxy acid formed on oxidation forms a lactone stable enough for titration (*cf.* Levene and coworkers (6)). The aldobionic acid itself does not lactonize, hence it is reasonable to infer that the carboxyl group formed by oxidation on the galactose half of the molecule yields a $<1,4>$ lactone.

9. Carbon atom 5 is eliminated since the methylglucosides formed at 25° and 100° are both hydrolyzed at the same rate by acid, and far too slowly for γ-glucosides (*cf.* Levene and Raymond (7)). The glucosides therefore appear to contain the $<1,5>$ oxidic ring. That they represent the α and β forms is indicated by the large difference between their molecular rotations, namely 30,000.

10. On the basis of these considerations the crystalline aldobionic acid is best formulated as α (or β)-glucurono-3 (or 6)-α-galactose.

EXPERIMENTAL.

1. Cinchonidine Aldobionate.—3 gm. of the calcium aldobionate described in the first paper ((1), p. 854) were treated with the theoretical amount of oxalic acid, the calcium oxalate was removed, and to the aldobionic acid solution was added an alcoholic solution of 1 equivalent of cinchonidine. The alcohol was removed under reduced pressure, a small amount of cinchonidine in excess was filtered off, and the solution concentrated *in vacuo* until the salt began to separate. It formed rosettes of needles. After standing overnight these were filtered off. An additional amount was obtained by adding acetone to the filtrate. The salt was recrystallized from water, washed with a little cold methyl alcohol, and dried.

0.1888 gm., dried to constant weight *in vacuo* over boiling CCl_4, lost 0.0088 gm.

Calculated for $C_{12}H_{20}O_{12} \cdot C_{19}H_{22}ON_2 \cdot 2H_2O$. H_2O, 5.25 per cent. Found. H_2O, 4.66 per cent.

0.0478 gm. anhydrous salt, 5 cc. in H_2O, $l = 1$, $\alpha = -0.62°$. $[\alpha]_D = -64.9°$.

0.00374 gm. anhydrous salt, by the Shaffer-Hartmann micro method (8), gave 1.06 mg. reducing sugars, calculated as glucose. Theory, 1.03 mg.

1.4548 gm. anhydrous salt in 50 cc. H_2O, required 21.8 cc. 0.101 N $Ba(OH)_2$, with phenolphthalein as indicator.

Calculated. 2.24 cc. N $Ba(OH)_2$. Found. 2.20 cc.

8.212, 7.450 mg. anhydrous salt gave 0.316, 0.275 cc. N_2(745.5 mm., 28.0°; 761.8 mm., 22.5°).

Calculated for $C_{31}H_{42}O_{13}N_2$. N, 4.31 per cent. Found. N, 4.28, 4.27 per cent.

The anhydrous salt melts at 172° (uncorrected) with decomposition.

2. Crystalline Aldobionic Acid from the Cinchonidine Salt.—The solution of the barium salt obtained in the titration of the cinchonidine salt was filtered from the insoluble cinchonidine, freed from barium with sulfuric acid, and concentrated *in vacuo.*

0.781 gm. acid (calculated as anhydrous), 15 cc. in H_2O, $l = 2$, α, final = $-0.86°$. $[\alpha]_D = -8.3°$.

A slight flocculent residue was filtered off, the alcohol was evaporated from the filtrate *in vacuo,* and the residue taken up in a small amount of water. Acetone was then added until the solution became turbid. After 24 hours in the ice box crystals began to form and a day later 0.25 gm. of small spindle-shaped needles was obtained.

0.2436 gm., dried to constant weight *in vacuo* at 100° over P_2O_5, lost 0.0238 gm.

Calculated for $C_{12}H_{20}O_{12} \cdot 2H_2O$. H_2O, 9.19 per cent. Found. H_2O, 9.77 per cent.

The air-dry acid melted at 116°, effervescing when the temperature was raised to about 128°.

3. Direct Preparation of Aldobionic Acid from the Specific Gum.—200 gm. of specific gum arabic (1) were dissolved in 2 liters of normal sulfuric acid and refluxed for 2 hours. The solution was cooled and the sulfuric acid removed with barium hydroxide. The filtrate was concentrated to 500 cc. and neutralized to phenol-

phthalein with barium hydroxide. The barium salt was precipitated by adding 2 volumes of alcohol and the gummy precipitate was taken up in water and reprecipitated twice with alcohol to remove galactose. The precipitate was dissolved in water, made up to a definite volume and barium was determined in an aliquot. The solution was then freed from barium by adding the theoretical amount of sulfuric acid, and was concentrated to 150 cc. under reduced pressure. After seeding with crystals of aldobionic acid obtained from the cinchonidine salt, it was placed in a vacuum desiccator over calcium chloride. After 2 days a thick mass of microscopic crystals had formed. In subsequent preparations seeding was found to be unnecessary. The crystals were filtered off with the aid of suction, washed with 50 per cent alcohol, and dried with alcohol and ether. The aqueous mother liquor was converted into the cinchonidine salt, yielding 10 gm. of crystals from which additional amounts of crystalline acid were obtained. The total yield of air-dry crystalline aldobionic acid was 35 gm. The total amount of acid present in the hydrolysate calculated as dry aldobionic acid on the basis of the barium content of the salt was 64 gm., so that about 55 per cent of the acid present was obtained in crystalline form. The acid was recrystallized twice from 50 per cent alcohol, separating with 2 molecules of water of crystallization.

1.8368 gm. air-dry substance, dried to constant weight at 100° *in vacuo* over P_2O_5, lost 0.1695 gm.

Calculated for $C_{11}H_{19}O_{10}COOH \cdot 2H_2O$. H_2O, 9.19 per cent. Found. H_2O, 9.23 per cent.

0.3213 gm. air-dry acid required 8.1 cc. 0.1 N NaOH for neutralization to phenolphthalein in the cold.

Acid equivalent, 396.6. Calculated for $C_{11}H_{19}O_{10}COOH \cdot 2H_2O$, 392.2.

No more alkali was neutralized on boiling, showing that no stable lactone was present.

4.478, 4.527 mg. anhydrous acid gave 2.235, 2.325 mg. H_2O and 6.707, 6.813 mg. CO_2.

Calculated for $C_{12}H_{20}O_{12}$. C, 40.4 per cent; H, 5.7 per cent. Found. C, 40.8, 41.0 per cent; H, 5.6, 5.7 per cent.

0.1702 gm. anhydrous aldobionic acid required 4.86 cc. 0.1 N NaOH.

Acid equivalent, 350.5. Calculated for $C_{11}H_{19}O_{10}COOH$, 356.2.

0.1702 gm. anhydrous aldobionic acid required 9.62 cc. 0.1 N iodine for

aldose determination according to Willstätter and Schudel (9). Calculated for $C_{11}H_{19}O_{11}CHO$, 9.56 cc.

0.2003 gm. air-dry aldobionic acid (9.77 per cent H_2O) was dissolved in 10 cc. of water, placed in a 2 dm. polarimeter tube, and an observation taken immediately.

	α	$[\alpha]_D$ Air-dry.	$[\alpha]_D$ Anhydrous.
2 min after solution effected.	+0 42°	+10.50°	+11.60°
15 " " " "	+0.13°	+3.25°	+3.60°
30 " " " "	−0 06°	−1.50°	−1.66°
60 " " " "	−0.24°	−6.00°	−6.64°
16 hrs. " " "	−0.31°	−7.75°	−8.56°

The acid dried at 100° gave somewhat lower initial values of $[\alpha]_D$, but after 24 hours the reading was −8.50°.

1.01 gm. of air-dry aldobionic acid were neutralized with NaOH and made up to a volume of 10 cc. with water. The original rotation, −0.72°, did not change on standing. $[\alpha]_D$, calculated as anhydrous aldobionic acid, = −7.85°.

0.5 cc. of 6 N hydrochloric acid was added to 5 cc. of the above solution and the rotation observed immediately and at intervals for several days. The original reading, −0.69°, did not change, indicating that no lactone was formed. $[\alpha]_D$, calculated as above, = −7.80°.

The air-dry acid melted at 118–119° and began to decompose at 128°. The anhydrous acid behaved in a similar manner, although the melting point was not quite so distinct.

4. Hydrolysis of Aldobionic Acid.—5 gm. of aldobionic acid were dissolved in 25 cc. of normal sulfuric acid and refluxed in an all-glass apparatus for 20 hours. After removal of the sulfuric acid the sugar acids were converted into the barium salts, which were precipitated by pouring into 95 per cent alcohol and purified by dissolving in a small volume of water and reprecipitating twice with alcohol. The barium was removed with sulfuric acid and the filtrate poured into 2 volumes of alcohol. A small amount of neutral precipitate was removed. An aliquot of the supernatant liquid was titrated to determine the amount of acid present, and the calculated amount of cinchonine was added. The solution was concentrated to 10 cc. *in vacuo* and allowed to stand in the ice box. Clusters of needles separated and were recrystallized from 75 per cent alcohol and air-dried. The salt melted at 198°

(uncorrected). Cinchonine glucuronate is stated to melt at 204° (10). The aldobionic acid did not form a crystalline cinchonine salt under these conditions.

0.2860 gm. of the air-dry cinchonine salt was dissolved in 10 cc. of water, leaving an insoluble residue which was centrifuged off. After determining the optical rotation 5 cc. of the solution were titrated with barium hydroxide to determine the quantity of the salt actually present.

Required: 2.65 cc. 0.101 N $Ba(OH)_2$, equivalent to 0.1308 gm. of cinchonine glucuronate.

$\alpha = +7.06°$, $l = 2$. $[\alpha]_D = +135°$; $[\alpha]_D$ of cinchonine glucuronate = +138.6°, according to Neuberg (10).

The remainder of the solution of the cinchonine salt was then also titrated with 0.101 N barium hydroxide solution, requiring an additional 2.45 cc. The total, 5.1 cc., corresponded to 0.100 gm. of glucuronic acid. The solution was concentrated *in vacuo* to 10 cc. and the rotation taken.

0.100 gm. substance in 10 cc. H_2O. $\alpha = +0.67°$, $l = 2$. $[\alpha]_D = +33.5°$. $[\alpha]_D$ of glucuronic acid = +36.3° (11).

The *p*-bromophenylosazone was prepared from the above solution and formed light yellow needles very difficultly soluble in water and alcohol. Recrystallized from alcohol it melted at 199°, instead of 205–208° and was strongly levorotatory in alcohol-pyridine mixture (*cf.* (12), p. 220).

The hexose formed on hydrolysis of the aldobionic acid was recovered from the alcohol filtrate from the precipitation of the barium salts of the sugar acids in the hydrolysis mixture (see above). The solution was evaporated to dryness *in vacuo,* taken up in a little hot glacial acetic acid, and filtered. The sugar crystallized on cooling and was recrystallized twice from methyl alcohol. It melted at 165–166° (uncorrected) and gave an equilibrium value of $[\alpha]_D = +80.6°$, $c = 2$, whereas galactose melts at 168° and shows an end value of $[\alpha]_D = +80.5°$ ((12), pp. 16–17).

The phenylosazone melted at 182° (uncorrected), the value given for galactosazone being 184°.

0.0819 gm. of the phenylosazone in 5 cc. of alcohol-pyridine (2:3) gave a reading after 30 minutes of +0.55° and after 48 hours,

+0.28°, l = 0.5. $[\alpha]_D$, 30 minutes, = +67°, $[\alpha]_D$, final, = +34°. Levene and La Forge (13) give for galactosazone $[\alpha]_D$, immediate, = +80°; $[\alpha]_D$, 24 hours = +34°.

5. Oxidation of the Aldobionic Acid to the Dicarboxybionic Acid.—12.4 gm. of air-dry aldobionic acid were oxidized with barium hypoiodite according to the method of Goebel (14). The calcium salt separated from a small volume of water in amorphous form, but became crystalline on repeated slow crystallizations from warm water, forming aggregates of microscopic needles.

0.5578 gm. air-dry salt lost 0.1093 gm. at 100° *in vacuo* over P_2O_5.

0.1133 gm. gave 0.0295 gm. $CaSO_4$.

Calculated for $C_{12}H_{18}O_{13}Ca \cdot 6H_2O$. H_2O, 20.9 per cent; Ca, 7.72 per cent. Found. H_2O, 19.6 per cent; Ca, 7.66 per cent.

4.730, 4.055 mg. anhydrous salt gave 2.050, 1.800 mg. H_2O and 5.940, 5.105 mg. CO_2.

0.1345 gm., 5.625 mg. gave 0.0431 gm., 1.830 mg. $CaSO_4$.

Calculated for $C_{12}H_{18}O_{13}Ca$. C, 35.1 per cent; H, 4.4 per cent; Ca, 9.8 per cent. Found. C, 34.3, 34.3 per cent; H, 4.9, 5.0 per cent; Ca, 9.4, 9.6 per cent.

0.7886 gm. of air-dry calcium salt was dissolved in water, made up to 20 cc., and the rotation observed in a 2 dm. tube. No mutarotation took place. α = −1.80°. $[\alpha]_D$ = −22.83°. To 10 cc. of the solution was added 0.5 cc. of 6 N HCl and the rotation was observed in a 2 dm. tube.

Time.	α°	$[\alpha]_D$°	Time.	α°	$[\alpha]_D$°
1 min.	−2.13	−28.4			
3 "	−2.10	−27.9	4 hrs.	−2.62	−36.2
7 "	−2.11	−28.1	22 "	−3.29	−43.8
27 "	−2.16	−28.7	28 "	−3.39	−44.6
2 hrs.	−2.40	−32.0	48 "	−3.43	−45.1
3 "	−2.55	−33.9		Constant.	

9 cc. of the solution were neutralized at room temperature with barium hydroxide and then titrated hot to determine the lactone content.

Required: an additional 3.33 cc. of 0.1 N $Ba(OH)_2$. The solution contained 0.3380 gm. of the air-dry salt, or 0.652 millimols. 51 per cent of one of the carboxyl groups was therefore in the <1,4> lactone form.

6. Hydrolysis of the Dibasic Acid.—2 gm. of the air-dry crystalline calcium salt were dissolved in 50 cc. of N sulfuric acid and alcohol was added to complete precipitation of the calcium sulfate After boiling off the alcohol the solution was refluxed for 18 hours in an all-glass apparatus fitted with a trap containing barium hydroxide. The reducing value of the solution and the carbon dioxide evolved were as follows: Reducing sugars as glucose, by Shaffer-Hartmann method, 0.57 gm., corresponding to hydrolysis of 1.64 gm. of air-dry calcium salt. CO_2 evolved neutralized 1.08 cc. of N $Ba(OH)_2$ solution, corresponding to 0.21 gm. of glucuronic acid decomposed. 28 per cent of the glucuronic acid formed was decomposed as indicated by the carbon dioxide absorbed.

The solution was freed from sulfuric acid and the acids were then converted into the cadmium salts by boiling with cadmium carbonate. The solution was concentrated to 15 cc. and allowed to stand in the ice box. An amorphous precipitate formed which crystallized in round clumps of white needles after treatment with norit. After repeated recrystallizations from water, it gave no naphthoresorcinol test for -uronic acids.

0.1457 gm. air-dry salt lost 0.0250 gm. at 100° *in vacuo* over P_2O_5.

Calculated for $(C_6H_{11}O_7)_2Cd \cdot 6H_2O$. H_2O, 17 7 per cent. Found. H_2O, 17.2 per cent.

5 926, 5.680 mg. anhydrous salt, mixed with CuO, gave 2.650, 2.495 mg. H_2O and 6.080, 5.850 mg. CO_2. 5.187 mg. gave 1.277 mg. CdO.

Calculated for $(C_6H_{11}O_7)_2Cd$. C, 28.7 per cent; H, 4.4 per cent; Cd, 22.4 per cent. Found. C, 27.9, 28.1 per cent; H, 5.0, 4.9 per cent; Cd, 21.6 per cent.

0.1078 gm. of the cadmium salt oxidized with 1 cc. 1:1 HNO_3 gave 0.028 gm. mucic acid, m.p. 217° (uncorrected).

The salt was therefore cadmium galactonate.[1]

The mother liquor from the cadmium salts was decolorized with norit and freed from cadmium with hydrogen sulfide. The presence of glucuronic acid in the solution was indicated by the immediate reduction of Fehling's solution in the cold, the formation of a crystalline *p*-bromophenylosazone melting at 206° (uncorrected), and the isolation of crystals resembling potassium acid saccharate on oxidation with nitric acid. The amount was too small for analysis.

[1] Isolated in amorphous form by Fischer and Ruff (15).

7. *Oxidation of the Aldobionic Acid with Nitric Acid.*—1.2598 gm. of air-dry aldobionic acid were dissolved in 15 cc. of nitric acid (sp. gr. 1.15). The solution was concentrated to one-third of its volume on the steam bath and allowed to stand overnight. 0.3686 gm. of mucic acid separated. After several recrystallizations it melted at 220° (uncorrected).

4.550, 6.152 mg. gave 2.180, 2.815 mg. H_2O and 5.683, 7.678 mg. CO_2.
Calculated for $C_6H_{10}O_8$. C, 34.27 per cent; H, 4.80 per cent. Found. C, 34.1, 34.0 per cent; H, 5.4, 5.1 per cent.

The nitric acid filtrate was evaporated to dryness on the boiling water bath and the residue was dissolved in 2 cc. of water and neutralized with 40 per cent aqueous potassium hydroxide. On acidification with glacial acetic acid, potassium acid saccharate immediately began to crystallize. For analysis it was recrystallized several times from very small volumes of water.

0.0359 gm. gave 0.0121 gm. K_2SO_4.
Calculated for $COOK(CHOH)_4COOH$. K, 15.75 per cent. Found. K, 15.1 per cent.

8. *The α-Methylglucoside Methylaldobionate and Its Rate of Hydrolysis.*—0.9936 gm. of anhydrous aldobionic acid was dissolved in 10 cc. of dry methyl alcohol containing 0.5 per cent of hydrochloric acid, and heated in a sealed tube for 2 hours at 100°. The volume was then made up to 25 cc. with dry methyl alcohol. $\alpha = +0.71°$, $l = 1$. $[\alpha]_D = +17.9°$. The hydrochloric acid was removed with a suspension of silver carbonate in dry methyl alcohol and the filtrate was concentrated to small bulk *in vacuo*. As the glucoside ester did not crystallize, it was taken up in 20 cc. of water, forming a clear, neutral solution that did not reduce Fehling's solution. $\alpha = +1.14°$, $l = 1$. $[\alpha]_D = +22.80°$.

The above value of $[\alpha]_D$ was calculated after hydrolyzing 9 cc. of the solution by boiling for a moment with 12 cc. of 0.1 N sodium hydroxide. The back titration was 0.3 cc. of 0.1 N hydrochloric acid.

$0.384 \times 1.17 = 0.4493$, weight of glucoside ester in 9 cc. of solution.

After hydrolysis, $\alpha = +0.23°$, $l = 2$, volume = 21.3 cc. $[\alpha]_D = +5.34°$, rotation of the methyl glucoside sodium salt.

11.7 cc. of 0.1 N hydrochloric acid were added to liberate the

free acid from the sodium salt. $\alpha = +0.15°$, $l = 2$, volume = 33 cc. $[\alpha]_D = +5.72°$, rotation of the methylglucoside acid. The solution showed no reducing sugars with Fehling's solution.

10 cc. of the solution of the glucoside ester were made up to 50 cc., with addition of enough hydrochloric acid to make the solution 0.1 N. The rate of hydrolysis of the glucoside was determined on 1 cc. samples by the method of Levene and Raymond (7), the Shaffer-Hartmann micro method being used for reducing sugars. The results are shown in Table I.

9. Preparation and Hydrolysis of the β-Methylglucoside Methylaldobionate.—0.6346 gm. of anhydrous aldobionic acid was dissolved in 25 cc. of dry methyl alcohol containing 0.5 per cent of hydrogen chloride. The solution was allowed to stand at 25° and the rotation was followed in a 2 dm. tube. The change was from an initial reading of −0.72° to −3.01° at the end of 24 hours, corresponding to specific rotations of −14.2° and −59.3°. In another experiment a maximum $[\alpha]_D$ of −66.4° was obtained.

TABLE I.

Rate of Hydrolysis of Glucoside Formed at 100°.

Time at 100°.	Glucose.	Glucoside hydrolyzed (as glucose).	Per cent hydrolyzed.
min.	*mg.*	*mg.*	
0	0.20		
0.5	0.28	0.08	1.8
1	0.38	0.18	4.0
2	0.70	0.50	11.2
5	1.18	0.98	21.9
10	1.78	1.58	35.3
15	2.18	1.98	44.2
30	2.61	2.41	53.8
45	2.80	2.60	58.0
60	2.96	2.76	61.6
360	4.68	4.48	100

At the end of 24 hours the solution was treated with silver carbonate to remove the hydrochloric acid, evaporated to dryness *in vacuo*, and made up to a volume of 25 cc. with cold water. The rate of hydrolysis was determined as in the case of the other glucoside. The results are shown in Table II and the values obtained in both cases are plotted in Fig. 1.

TABLE II.

Rate of Hydrolysis of Glucoside Formed at 25°.

Time at 100°.	Glucose.	Glucoside hydrolysed (as glucose).	Per cent hydrolysed.
min.	*mg.*	*mg.*	
0	0.18		
0.5	0.42	0.24	2.0
1	0.70	0.52	4.3
2	1.06	0.88	7.4
5	2.80	2.62	21.8
10	4.34	4.16	34.7
15	5.50	5.32	44.3
30	5.70	5.52	46.0
45	7.75	7.57	63.1
60	8.00	7.82	65.2
120	8.95	8.77	73.1
180	9.35	9.17	76.4
360	12.20	12.02	100

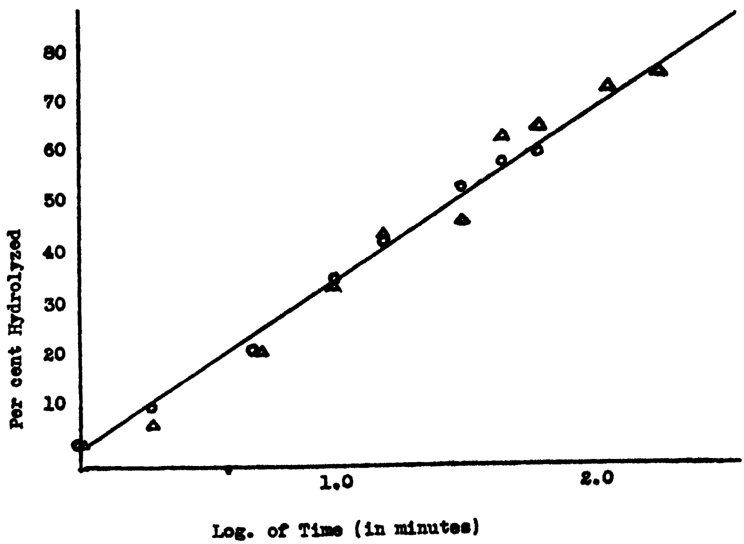


FIG. 1. Rate of hydrolysis of methyglucosides with 0.1 N HCl at 100°. The circles represent glucoside formed at 100°, $[\alpha]_D = +17.9°$; the triangles, glucoside formed at 25°, $[\alpha]_D = -59.3°$.

10. Isolation of a Lactone-Forming Acid from Hydrolysis Products of Specific Gum Arabic.—The mother liquors from the crystalline aldobionic acid recovered from about 600 gm. of the specific gum were neutralized in the cold with barium hydroxide solution to the first pink color with phenolphthalein. The barium salts were precipitated by pouring the solution into 95 per cent alcohol, and the alcoholic supernatant liquid was concentrated to small volume *in vacuo* and neutralized hot with barium hydroxide. The barium salt was precipitated with alcohol and washed thoroughly with methyl alcohol. The free acid was liberated with sulfuric acid and allowed to concentrate slowly in a vacuum desiccator. As no crystals were obtained, the residue was dissolved in water and made up to 25 cc. $\alpha = -0.33°$, $l = 1$. On titration with barium hydroxide 2.5 cc. of 0.4 N $Ba(OH)_2$ were required for neutralization immersed in ice water, and an additional 4.3 cc. at the boiling point, corresponding to 63 per cent of lactone.

The above solution of the barium salt was precipitated by pouring it into 8 to 10 volumes of alcohol and the salt was dried to constant weight at 61° *in vacuo* over P_2O_5.

0.3860 gm., 15 cc. in H_2O, $\alpha = -0.12°$, $l = 0.5$. $[\alpha]_D = -9.3°$.

2.573 mg. were equivalent to 0.55 mg. glucose (Shaffer-Hartmann micro method)

0.1242 gm. gave 0.0329 gm. $BaSO_4$

Calculated for $C_{21}H_{35}O_{18}CHO(COO)_2Ba$. Glucose, 21.7 per cent; Ba, 16.6 per cent. Found. Glucose, 21.4 per cent; Ba, 15.6 per cent.

The analyses thus indicate the barium salt of a dicarboxy-aldotetronic acid. On this basis $[\alpha]_D$ of the free acid becomes $\frac{-0.33° \times 25}{0.347 \times 4 \times 6.8} = -8.8°$, 6.8 being the total $Ba(OH)_2$ titration. The yield was therefore 0.94 gm.

11. Phenylosazone of the Aldobionic Acid.—1 gm. of air-dried aldobionic acid and 2 gm. of phenylhydrazine were dissolved in 3 cc. of 50 per cent acetic acid and heated in the steam bath for 1 hour. No osazone separated from the deep red-brown solution on cooling. 3 volumes of alcohol precipitated an amorphous yellow product which was dissolved in hot dry methyl alcohol. A brown amorphous precipitate separated on cooling and was discarded. To the filtrate 2 volumes of ether were added, precipitating a

deep yellow amorphous material. After drying to constant weight *in vacuo* at 61° it was analyzed for nitrogen.

3.553, 3.728 mg. gave 0.362, 0.377 cc. N_2 at 759.2, 759 mm. and 31.0°, 32.0°. N, 11.5, 11.3 per cent.

Calculated for $C_{24}H_{30}O_{10}N_4$. N, 10.5 per cent. For $C_{24}H_{30}O_{10}N_4 \cdot C_6H_5NHNH_2$. N, 13.1 per cent.

The product thus appears to be a mixture of the osazone and its phenylhydrazine salt.

SUMMARY.

1. Isolation of a crystalline aldobionic acid from the hydrolysis of specific gum arabic is described.

2. Experiments are described which identify the substance as a glucuronogalactose and permit a more precise provisional formulation as α(or β)-glucurono-3(or 6)-α-galactose.

In conclusion the writers wish to express their gratitude to Dr. P. A. Levene for his valuable suggestions, and to Dr. Walter A. Jacobs for facilities extended.

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THE HEAT OF COMBUSTION OF ERGOSTEROL, ISOERGOSTEROL, AND CHOLESTEROL.

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In connection with studies on vitamin D we have had occasion to compare the heats of combustion of unirradiated ergosterol, isoergosterol, and cholesterol. The literature, apparently, contains no reference to determinations of these values.

The ergosterol was purified by crystallizing crude yeast sterol five times from alcohol-benzene, 2:1. It gave $[\alpha]^{25}_{5461} = -157°$ in $CHCl_3$ (Specimen A-B of Bills and Cox (1929)). The isoergosterol, $[\alpha]^{25}_{5461} = -53°$, was prepared from the ergosterol by treatment with hydrochloric acid according to the method of these authors. The cholesterol was Specimen E of Bills, Honeywell, and MacNair (1928), purified by subjecting ordinary cholesterol three times to bromination and debromination. We crystallized it again from alcohol in order to remove any decomposition products that might have developed during the 2 years since it was prepared.

The three sterols were dehydrated in a high vacuum at 80° for 15 minutes. Immediately afterwards the crystals were compressed into pellets for combustion. We used a Parr adiabatic oxygen bomb calorimeter, and made three determinations with ergosterol, two with isoergosterol, and three with cholesterol. The results are summarized in Table I.

From Table I it is evident that ergosterol suffers no considerable change in its heat of combustion when it undergoes transformation into isoergosterol. The very small difference observed, 10,053 as against 10,050 calories per gm., is well within the narrow limits of experimental error. The heat of combustion of cholesterol was 10,289 calories per gm., or 236 calories per gm. more

than that of ergosterol This difference merely reflects the fact that cholesterol contains 4 more hydrogen atoms than ergosterol The obvious conclusion from these facts is that the molecular

TABLE I

Heats of Combustion of Ergosterol, Isoergosterol, and Cholesterol

Substance	Weight of pellet	Calories$_{15}$ per gm	Deviation from mean	Kilo calories$_{15}$ per mol
	gm		*per cent*	
Ergosterol	0 4674	10035	0 2	
"	0 5474	10066	0 1	
"	0 5878	10058	0 1	
Mean		**10053**		**3844**
Isoergosterol	0 2451	10054	0 0	
"	0 3313	10045	0 1	
Mean		**10050**		**3843**
Cholesterol	0 3354	10287	0 0	
"	0 4810	10274	0 2	
"	0 4683	10307	0 2	
Mean		**10289**		**3978**

structure responsible for the phenomenal activatability of ergosterol is not associated with an anomalous heat of combustion

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A STUDY OF GLUTATHIONE.

I. ITS PREPARATION IN CRYSTALLINE FORM AND ITS IDENTIFICATION.

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Knowledge of the chemical nature of those agents which modify and control physiologic processes is slowly gathered by extended investigations over years of time and in many laboratories. This has been true of epinephrine, thyroxine, insulin, and glutathione. The essential contributions in each case furnished definite chemical evidence concerning the respective compounds; additions and modifications have then been made. This paper is an addition to our knowledge of glutathione; it in no way alters the original fundamental work of Hopkins.

Theoretic Considerations.

An investigation concerning the chemical and physiologic properties of glutathione was begun in our laboratory in 1924. At that time, several gm. of glutathione were prepared according to the method of Hopkins (5). In order to continue this investigation, it became necessary to isolate more of the material and the first steps toward this end were made in the early part of 1928. After the first description of a method for the separation of glutathione by Hopkins (5), papers were published by Hunter and Eagles (6) and by Johnson and Voegtlin (7). Hunter and Eagles slightly modified the method of Hopkins, but the yield of glutathione was not quite so high and the final product contained a higher percentage of nitrogen and less sulfur than glutamyl cysteine. Johnson and Voegtlin separated glutathione in much lower yield,

TABLE I.

*Yield of Glutathione.**

Lot No.	Weight.	Date.	Glutathione, not crystalline.	Lot No.	Weight.	Date.	Glutathione, not crystalline.	Glutathione, crystalline.
	kg.	*1928*	*gm.*		*kg.*	*1929*	*gm.*	*gm.*
1	22	Aug. 22	2.0	25	45	Jan. 31	0.0	
2	22	" 28	1.0	26	45	Feb. 5	26.4	
3	22	Sept. 4	0.0	27	45	" 12	42.0	
4	45	" 18	8.0	28	45	" 19	15.6	
5	45	" 25	11.3	29	45	" 26	15.6	
6	45	Oct. 2	11.0	30	45	Mar. 5	15.5	
7	45	" 9	6.1	31	45	" 13	16.5	
8	45	" 16	12.5	32	45	" 20	16.8	
9	45	" 23	0.0	33	45	" 26	24.9	
10	45	" 30	23.2	34	45	Apr. 2	14.1	
11	45	Nov. 6	7.8	35	45	" 9	19.6	
12	45	" 13	3.2	36	45	" 16	21.5	
13	45	" 15	11.9	37	45	" 23	24.7	
14	45	" 20	13.6	38	45	" 30		
15	45	" 27	37.0	39	45	May 7	22.0	6.6
16	45	Dec. 4	16.3	40	45	" 14	17.0	5.1
17	45	" 11	19.3	41	45	" 22		7.1
18	45	" 18	0.0	42	45	" 29	90.0	14.9
19	45	" 26	0.0	43	45	June 4		11.1
		1929		44	45	" 11		12.8
20	45	Jan. 2	2.5	45	45	" 18		5.4
21	45	" 9	12.2	46	45	" 25		10.5
22	45	" 15		47	45	July 2		5.0
23	45	" 23	14.6					
24	45	" 29						

* The loss of glutathione in Lot 3 was due to the addition of large amounts of uranium acetate. Lot 9 was heated at 100° in a steam kettle with a large amount of barium acetate; glutathione could not be separated after this treatment. Glutathione was lost in Lots 18 and 19 through adsorption on aluminum hydroxide cream. Lots 21, 22, 23, and 24 were heated by passing through a block tin pipe. No glutathione was isolated from Lot 25 because insufficient toluene was added.

but their analysis agreed better with the theoretic values for glutamyl cysteine. It was decided, therefore, to follow the method of Johnson and Voegtlin, to use a large centrifuge recommended by them. and to treat 45 kilo lots of bakers' yeast at a time.

In addition to the separation of glutathione, the total amount of the thiol compound present in the yeast and in each of the solutions obtained during the isolation was determined. The quantitative study of each step of the method of isolation with each lot of yeast was of great help. The results are summarized in Table I, which is made clearer by the following paragraphs.

Temperature and Duration of the Initial Heating of the Yeast Suspension.—Lots of yeast, from Lots 1 to 24 inclusive, were heated. 45 kilo lots of yeast were suspended in 70 liters of tap water and were heated for various lengths of time. The results showed that prolonged heating at or near the boiling point carried a large amount of material into solution, and although the apparent thiol content was high, it was invariably lost by adsorption during the precipitation of the impurities. This was particularly true of Lot 3 in which all glutathione was removed from solution before the final precipitation with absolute alcohol.

In order to reduce the time of heating to a minimum, the suspension of 45 kilos of yeast in 130 liters of water was rapidly passed through a coil of block tin pipe, 10 mm. in diameter and 9 meters long. The coil was placed in a container holding 30 liters of water which was kept boiling with a steam coil. The suspension of yeast cells was poured from the end of the coil directly on ice which was placed in 75 liter crocks. The temperature of the solution as it left the coil was about 80° and the time required for the solution to pass from the inlet to the outlet of the coil was 25 seconds. By this method of heating, the temperature of the solution was raised from 20° to 80° and back to 15° within less than 60 seconds. The thiol content of the solution was the same as that of suspensions which were heated longer. The solutions were much more easily treated for the separation of glutathione.

The work of Raymond (9) on the coenzyme of yeast showed that toluene liberated the coenzyme from yeast cells. If toluene at room temperature could liberate glutathione from the yeast cell, then the amount of impurities carried into solution would be reduced to the minimum. Toluene was tried and found to liberate all the glutathione from the yeast cell. Titration of the thiol group, either with iodine or with potassium ferricyanide, indicated the presence of even more glutathione than that in similar solutions which were heated. The action of toluene will

be more fully described in another report, but it is now evident that in the isolation of crystalline glutathione from yeast, the use of a hydrocarbon to liberate the thiol compound is one of the most important modifications of Hopkins' method. Lots 25 and 26 were treated with toluene. All lots from Lots 27 to 47 inclusive were treated with benzene, which was shown to have the same effect as toluene.

Volume of the Suspension of Yeast.—Hopkins originally recommended a suspension of 45 kilos of yeast in 10 liters of water. This was repeated three times. Johnson and Voegtlin used 45 kilos of yeast in 100 liters of water and reextracted with 50 liters of water. In Lots 4 to 10, 45 kilos of yeast were suspended in 70 liters of water, but after it was shown that the glutathione lost in the extracted cells was inversely proportional to the total volume, 45 kilos of yeast were suspended in 210 liters of water; the extracted cells were not reextracted; not more than 15 per cent of the total glutathione was lost in the extracted cells.

Precipitation of Glutathione as the Lead Salt.—The first separation of glutathione by all investigators has been precipitation as the lead salt. Five factors have been varied: (1) the volume of the solution, (2) the pH of the solution, (3) the amount of lead salt added, (4) the time which the solution stands before separation of the lead precipitate from the solution, and (5) the amount of extractives from the yeast cell.

The lead salt is so insoluble that the volume of the solution is immaterial. Lots of yeast from Lots 10 to 47 were extracted with a total volume of 210 liters to which the lead acetate was added.

If the pH of the solution is increased to 9.5 or 10, a water-white filtrate may be obtained and all reducing substances are precipitated. If the suspension of yeast is heated to liberate the glutathione, a heavy precipitate is produced by lead acetate. A much smaller precipitate is formed when lead acetate is added to the extract of yeast made with cold water and benzene; the pH of such a solution is about 5.5. At this pH, about 85 to 90 per cent of the glutathione in solution is precipitated. Any increase in the pH carries down impurities which cause subsequent losses of glutathione.

The amount of lead salt necessary to give maximal precipitation of glutathione depends on the amount of impurities. For Lots 10

to 47, we have used 2 kilos of lead acetate for the extract of 45 kilos of yeast.

Hopkins allowed the solution to settle before separation of the lead salt, probably not more than an hour. Johnson and Voegtlin removed the lead salt after the solution had stood overnight. We have found that the precipitation of the glutathione is complete within less than 5 minutes. The lead precipitate was therefore removed by centrifugation of the solution without delay after the addition of the lead acetate. After precipitation in this way, the solution contains appreciable amounts of thiol compounds but the precipitate contains a much smaller percentage of compounds which have to be removed by subsequent steps.

The amount of total extractives in the solution has an important bearing on the completeness of the precipitation of glutathione with lead. If the suspension of yeast is heated, a large amount of material goes into solution and precipitation with lead acetate is less complete.

Adsorption of Glutathione on Precipitates Formed with Aluminum Hydroxide Cream, Uranium Acetate, Barium Phosphotungstate, and Barium Sulfate.—In order to remove protein material as much as possible before the addition of lead acetate, aluminum hydroxide cream, 1.0 per cent, was added to the extract of the yeast. Aluminum hydroxide cream at pH 5 removes protein material from the solution and carries down almost no glutathione, but at pH 7.4 this reagent may remove glutathione almost completely from solution. The loss of all the glutathione in Lots 18 and 19 showed this reagent to be useless and it was not added to any of the other preparations.

Hopkins proposed the use of uranium acetate to remove impurities from the solution prepared from the lead precipitate with sulfuric acid. If the yeast suspension is heated and a large amount of material is extracted from the yeast, the addition of uranium acetate to the solution will precipitate a large amount of impurities, but this separation is attended with great loss of glutathione. In a slightly acid solution, uranium acetate will not precipitate glutathione, but as the pH is increased, the amount of glutathione carried down increases: it is possible to precipitate all glutathione at this step. In Lot 3 all glutathione was lost because of the addition of too much uranium acetate to the solution. The qualitative

test for uranium with potassium ferrocyanide indicates the presence of uranium long before the maximal precipitation of impurities with uranium acetate is reached. It was subsequently shown that uranium acetate does not remove impurities which phosphotungstic acid does not remove; uranium acetate was not used in Lots 4 to 47.

Quantitative determination of glutathione after treatment with phosphotungstic acid showed that under proper conditions, no glutathione is precipitated, but that barium phosphotungstate at pH 7.4 to pH 8.0 carries down large amounts of glutathione. This loss is unavoidable and is best controlled by reducing to a minimum the amount of impurities which are removed with phosphotungstic acid.

Large amounts of glutathione may be adsorbed on barium sulfate and removed from solution. This is particularly true if the pH is 7.4 or above. The best yields of glutathione are obtained if both sulfuric acid and barium salts are used in minimal amounts.

Decomposition of Lead Precipitate with Sulfuric Acid.—Hopkins decomposed the lead salt of glutathione with 0.5 N sulfuric acid by grinding in a mortar. Johnson and Voegtlin carried out this step in the same way. This method is so time-consuming that we have modified it. The lead precipitate is suspended in 5 liters of water containing the sulfuric acid and is vigorously stirred with a nickel stirrer. The maximal decomposition of the lead salt is obtained in the minimum of time.

Precipitation of Impurities with Phosphotungstic Acid.—For the precipitation of impurities not removed with uranium acetate, Hopkins used phosphotungstic acid. Hunter and Eagles and Johnson and Voegtlin also have used phosphotungstic acid at this step. We have used both tungstic acid and phosphotungstic acid. Tungstic acid is objectionable since it does not precipitate as many impurities as does phosphotungstic acid, and its use introduces sodium sulfate into the solution.

Two important observations were made concerning the use of phosphotungstic acid: (1) a large amount of material is precipitated by phosphotungstic acid at 0° which is soluble at 20°; not only is it necessary to cool the solution to 0° during this step but the solution must be filtered at 0° to prevent resolution of the impurities, and (2) the limited solubility of the phosphotungstic acid salt

of glutathione at 0° requires this step to be carried out in a large volume (from 8 to 10 liters).

It was not found necessary to have the solution 0.5 N with respect to sulfuric acid during the precipitation with phosphotungstic acid. This reagent does not precipitate glutathione in a solution 0.1 N with respect to sulfuric acid, and the impurities are as completely removed. Reducing the sulfuric acid content to about 4 times the equivalent of glutathione minimizes the loss of glutathione with the barium sulfate which results from the addition of barium hydroxide used in the removal of the excess phosphotungstic acid.

Precipitation with Mercury.—No essential change was found necessary to give the maximal precipitation. The mercury sulfate solution was changed slightly to reduce as far as possible the amount of sulfuric acid present. The mercury precipitate was filtered immediately, as it was found that the precipitate that slowly separates from the solution contains more impurities than it does glutathione. The mercury precipitate was suspended in distilled water and thoroughly agitated with a mechanical glass stirrer to disintegrate it completely before treatment with hydrogen sulfide. The precipitation of glutathione with mercury sulfate is remarkably complete; only traces of the thiol compound are left in the filtrate.

The steps during which sulfuric acid was removed, the solution concentrated to small volume, and the glutathione precipitated with absolute alcohol and ether were not changed.

Yield of Glutathione.—When yeast is treated as outlined, about 23 gm. of glutathione may be separated from 45 kilos of yeast. This is the average of Lots 39 to 47 inclusive. Determination of the total thiol compounds present indicates that the total amount of glutathione in the form as separated is approximately 45 gm. in 45 kilos of the yeast which was used.

Chemical Properties of Glutathione.—Hopkins described glutathione as a compound containing carbon, hydrogen, oxygen, nitrogen, and sulfur, easily soluble in water and insoluble in ethyl alcohol and other organic solvents. Its nitrogen and sulfur content were 11.70 and 12.31 per cent, respectively. After hydrolysis, he separated glutamic acid in 88 per cent yield as the hydrochloride and cystine in 60 per cent yield. He suggested the structure to be glutamyl cysteine.

The product obtained by Hunter and Eagles contained too much nitrogen and too little sulfur to be glutamyl cysteine and they therefore suggested a more complex structure for glutathione but were unable to secure satisfactory chemical evidence for their hypothesis. Johnson and Voegtlin separated glutathione, the composition of which agreed more nearly with that prepared by Hopkins; its composition was much closer to that of glutamyl cysteine. Benedict and Newton (2) recently found that glutathione prepared from blood contains too much nitrogen and too little sulfur to be glutamyl cysteine.

Analysis of the glutathione which we have prepared gave about 13 per cent of nitrogen and about 11 per cent of sulfur. Titration of the SH group in alcohol with iodine or with potassium ferricyanide electrometrically showed that the material contained about 75 per cent of the theoretic amount of the SH group required for glutamyl cysteine.

Fractional precipitation with lead acetate at varying pH, or fractional removal of phosphotungstic acid precipitates in a concentrated solution, or fractional precipitation with increasing amounts of copper hydroxide or mercury sulfate did not bring about any significant change in either the nitrogen or sulfur content. The material appeared to be homogeneous.

By the time we had treated thirty-nine lots of yeast, of 45 kilos each, the method as outlined had been developed to a point at which so little extraneous material was present that the thiol compound could assert its physical properties. In short, the solution of Lot 40 which was prepared for the precipitation of the glutathione with absolute alcohol set to a mass of crystals just before the alcohol was added. The crystals were washed with glacial acetic acid, and then with absolute alcohol in a centrifuge tube, and were filtered on a Buchner funnel. 5 gm. were obtained. Lots 39 and 41 were then carried through in the same way but the solution was seeded with some of the crystals. 13 gm. of crystals were separated. Six more lots of yeast of 45 kilos each were treated and the crystals were separated from each lot; a total of 78 gm. of crystalline glutathione has been isolated. (See Fig. 1.)

Identification of Crystalline Glutathione.—It was soon shown that the crystals contained a thiol group which was equivalent to 81 per cent of the theoretical amount in glutamyl cysteine: this

indicated a molecular weight of 307. It was next shown that the percentage composition indicated the presence of 3 atoms of nitrogen for each atom of sulfur. These results pointed to the probability that the crystals were a tripeptide and the difference in molecular weight (57) between glutamyl cysteine and the tripeptide indicated the probable presence of glycine. Hydrolysis with hydrochloric acid confirmed this.

The hydrochloric acid was removed in a vacuum, the cysteine was precipitated with mercury sulfate. The excess mercury was

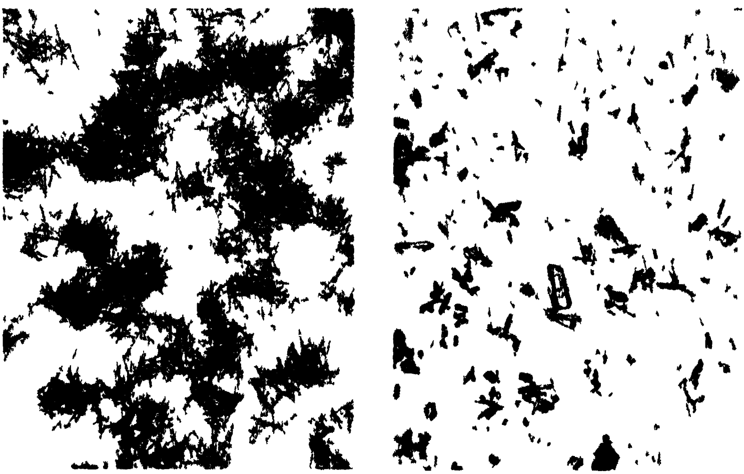


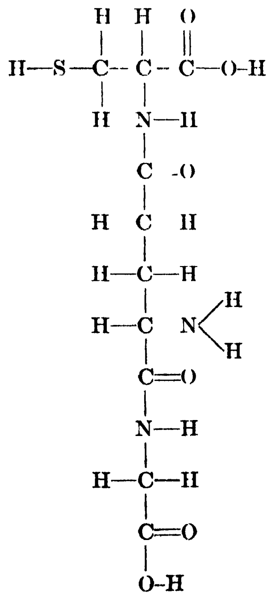
Fig 1 Crystalline glutathione On the left are crystals of the tripeptide as it separates from the original solution On the right are the recrystallized tripeptide crystals The crystals are coarser and of much greater cross-section. × about 16

removed as sulfide and the solution was concentrated and benzoylated. Hippuric acid was separated in 75 per cent of the theoretic amount. Another portion of crystals was hydrolyzed and glutamic acid was separated as its hydrochloride in a yield of 66 per cent of the theoretic amount.

The mercury precipitate of cysteine was decomposed with hydrogen sulfide, and the solution was concentrated to small volume. 95 per cent alcohol was added and the thiol group present was titrated with iodine. 94 per cent of the theoretic amount was

required. The alcohol was removed and cystine was separated in pure crystalline form by the addition of sodium acetate. The yield of crystals was 76 per cent of the theoretic amount. Nitrogen (Kjeldahl method) and sulfur (Parr bomb) determination showed 13.68 and 10.42 per cent respectively. These results leave no doubt that the crystals are a tripeptide of glutamic acid, glycine, and cysteine. They do not show the manner in which the three amino acids are combined.

3.07 gm. of the crystals were treated with nitrous acid and the solution was then refluxed for 18 hours after the addition of concentrated hydrochloric acid. Glycine was separated as hippuric acid in the same yield as before but no glutamic acid could be isolated. This is evidence that the glycine is attached through its amino group and not through its carboxyl group, also that the amino group of the glutamic acid is not substituted. The reaction toward nitrous acid does not show whether the glycine is attached to the carboxyl group of the glutamic acid or to the carboxyl group of the cysteine.



The structural formula tentatively suggested for the tripeptide, glutathione, glutamyl-glycine-cysteine.

Evidence for this was secured by the action of hydrogen peroxide in the presence of ammonia. Carbon dioxide was given off and after hydrolysis neither glutamic acid nor glycine could be isolated, but succinic acid was separated. This shows that probably the amino group of the glycine is attached to the carboxyl group of the glutamic acid which is next to the amino group in the glutamic acid. The crystals are therefore glutamyl-glycine-cysteine.

After it has been shown that 15 gm. of the tripeptide can be separated in crystalline form from 45 kilos of yeast the question immediately arises as to whether cysteine is present in any other form. Further work on this question is necessary but analysis of the material precipitated from the mother liquor of the crystals with absolute alcohol shows it to contain nitrogen and sulfur in amounts which are almost theoretic for the tripeptide. Glycine can also be separated in the same yield as from the crystalline tripeptide after hydrolysis with hydrochloric acid. These results make it highly probable that practically all of the cysteine is in the form of the tripeptide.

The results obtained by Hopkins on the identification of glutathione leave no doubt that the substance which he isolated was glutamyl cysteine. The formation of this dipeptide from the tripeptide is an obvious explanation of the source of the dipeptide. Preliminary experiments indicate that the amount of the tripeptide which can be isolated greatly diminishes if the yeast extract is allowed to stand before the addition of lead acetate. Further work on the stability of the tripeptide is in progress. It has been clearly shown that the crystalline tripeptide can be heated to 100° in water or in 0.1 N acid for several hours without loss of glycine but after the crystals were heated in water at this temperature, subsequent crystallization of the material was greatly retarded and the weight of crystalline material recovered was much less than that from a similar solution which was heated only to 60°.

EXPERIMENTAL.

Method for Isolation of Crystalline Glutathione from Yeast.—45 kilos of bakers' yeast, free from starch, are broken into small lumps and evenly distributed between four 75 liter crocks, each of which contains 50 liters of distilled water. The suspension of yeast is thoroughly stirred with a mechanical stirrer. 2400 cc.

of benzene are added to each jar and the suspension is again vigorously stirred. 15 liters of distilled water are added to each jar and the solution is allowed to stand about 2 hours. 210 cc. of concentrated sulfuric acid dissolved in 4 liters of distilled water are added to each jar and the solution is again stirred. 1200 gm. of crystalline barium hydroxide containing 8 molecules of water dissolved in 3 liters of hot water are added to each crock. The solution is stirred and centrifuged immediately in a Sharples supercentrifuge. The precipitation of barium sulfate greatly facilitates the removal of the extracted yeast cells. The extract of the yeast is about 210 liters at this time; it is slightly turbid. It is again divided evenly among four crocks. 500 gm. of neutral lead acetate dissolved in 2 liters of distilled water are added to each crock. The pH of the solution is approximately 5.5. After thorough mixing the solution in the four crocks is centrifuged. The lead precipitate is suspended in 4 liters of water to which are added 900 cc. of 5 N sulfuric acid. This step is carried out in a 6 liter glass museum jar. The suspension of the lead salt is vigorously stirred with a nickel stirrer for 2 hours until it is reduced to a smooth homogeneous creamy consistency without lumps. This step is not time-consuming, as the agitation is entirely mechanical. The solution is filtered and made to a volume of 7.5 liters. Barium hydroxide is added until the pH of the solution is 4. The amount of barium hydroxide required varies between 600 and 700 gm. The barium precipitates a large amount of material not glutathione. This is removed by filtration. Sulfuric acid is then added until the barium hydroxide is just neutralized and then 150 cc. of 5 N sulfuric acid are added in excess. This acidity is about equivalent to four times the glutathione present. The solution is filtered in order to remove the barium sulfate which interferes with the rapid filtration which is required at the next step. The solution is cooled to 0° in an ice and salt pack and phosphotungstic acid is added until no further precipitation is produced. Between 45 and 50 gm. are required. The solution is filtered while the temperature is maintained as close to 0° as possible. Barium hydroxide is added to the filtrate until the pH is 7.0. The solution is again filtered. This removes the excess phosphotungstic acid. The barium is removed with sulfuric acid. Approximately 200 cc. of 5 N sulfuric

acid are required. Barium sulfate is filtered out and the glutathione is precipitated with mercury sulfate. From 200 to 350 cc. of the modified Hopkins' solution of mercury sulfate[1] are required. This reagent is added until no more precipitate forms. The mercury precipitate is filtered, is broken up with a glass mechanical stirrer, and is then decomposed with hydrogen sulfide. The solution is filtered from mercury sulfide and after being cooled barium hydroxide is added until the pH is 7.5. The solution is treated with hydrogen sulfide to precipitate heavy metals. It is filtered and the barium is exactly removed with sulfuric acid. The barium sulfate is removed by filtration. The solution is evaporated to about 30 or 40 cc., is placed in a wide, deep container, and is allowed to stand after the addition of a small amount of the tripeptide previously isolated. The solution sets to a firm crystalline mass during the next few hours. The crystals are suspended in glacial acetic acid, then transferred to 50 cc. centrifuge cups, and separated from the solution by short centrifugation. They are then washed with glacial acetic acid and again centrifuged. They are washed out of the centrifuge cup with absolute alcohol and are filtered on a Buchner funnel.

The compound is purified by recrystallization from water.

Crystallization of the Tripeptide.—11 gm. of the crude material were suspended in 25 cc. of water. This was not sufficient to dissolve the crystals. The solution was heated to 60° over a free flame and was stirred with a thermometer. It was filtered through paper on a small Buchner funnel, placed in a small beaker, and put in the ice box after the addition of a few crystals of the tripeptide. After 2 days the crystalline mass was broken up and the solution was again placed in the ice box for 24 hours. The crystals were then filtered off, washed with glacial acetic acid, and finally with absolute alcohol. After drying in the desiccator, the weight was 6.4 gm.

Hydrolysis of the Tripeptide, Separation of Glutamic Acid.—2 gm. of Lot 40 were dissolved in 100 cc. of 25 per cent hydrochloric acid and the solution was refluxed for 8 hours. It was then evaporated in a vacuum to 10 cc.; the solution was saturated with hydrochloric acid gas at 0°. On the following day, 620 mg. of

[1] 200 gm. of mercury sulfate, 50 cc. of 10 N sulfuric acid, 40 cc of concentrated sulfuric acid, and 600 cc. of water are mixed in the order given.

crystals were filtered from the solution. The filtrate was again concentrated and saturated with hydrochloric acid gas at 0°. 170 mg. more of the crystals were separated. Finally a crop of 20 mg. were separated. The total weight was 710 mg. After recrystallization, the melting point was found to be 201°. When mixed with glutamic acid hydrochloride, the melting point was unchanged.

0.1524 gm. substance: 8.35 cc. 0.1 N NH_3. Calculated. N 7.63. Found. 7.66.

0.1534 gm. substance: 8.42 cc. 0.1 N NH_3. Found. N 7.67.

This is a yield of glutamic acid hydrochloride equivalent to 66 per cent of the theoretic amount.

Separation of the Benzoyl Derivative of Glycine.—2 gm. of the recrystallized tripeptide were refluxed in 100 cc. of constant boiling hydrochloric acid for 15 hours. The solution was evaporated to dryness in a vacuum in order to remove the hydrochloric acid. The residue was dissolved in 150 cc. of water and mercury sulfate was added until precipitation was complete. The precipitate was filtered out. The mercury was removed from the filtrate with hydrogen sulfide; the solution was concentrated to 50 cc. 50 cc. of 5 N sodium hydroxide were added and 5 cc. of benzoyl chloride. This solution was shaken until the odor of benzoyl chloride was very slight. Hydrochloric acid was added and the solution was extracted four times with 20 cc. portions of ethyl acetate. The ethyl acetate was removed in a vacuum and the residue was dissolved in chloroform. Hippuric acid (1) separated almost immediately. After 24 hours, 880 mg. were filtered off. The melting point was 188°.

Separation of Cystine.—The mercury precipitate which was removed before the isolation of benzoyl glycine was decomposed with hydrogen sulfide and the mercury sulfide was removed by filtration. The solution was evaporated to dryness in a vacuum, and was dissolved in 50 cc. of 95 per cent alcohol and oxidized with iodine dissolved in alcohol. 780 mg. were required. This is 94 per cent of the theoretic amount. The alcohol was removed by evaporation in a vacuum and the residue was dissolved in 50 cc. of water. 5 gm. of sodium acetate in 25 cc. of water were added. After 24 hours, 596 mg. of cystine were filtered from the solution. This is a yield of 75 per cent. The cystine was identified by its

quantitative determination by a modification of the Folin-Looney method. 50 mg. in 50 cc. of 0.1 N sulfuric acid gave a colorimetric reading of 20:20 with a solution of an equal amount of pure cystine.

Ultimate Analyses of the Tripeptide.

0.149 gm. substance: 0.2104 gm. CO_2 and 0.0764 gm. H_2O.
$C_{10}H_{17}O_6N_3S$. Calculated. C 39.06. Found. 38.47.
H 5.58. " 5.63.

0 1503 gm. substance: 0.1151 gm. $BaSO_4$. Calculated. S 10.42. Found. 10.46.

0.1635 gm. substance: 0.1239 gm. $BaSO_4$. Found. S 10.35.

0.1565 " " : 15.29 cc. 0.1 N NH_3. Calculated. N 13.68. Found. 13.67.

0.1535 gm. substance: 15.36 cc. 0.1 N NH_3. Found. N 14.01.

Melting Point of the Tripeptide.—The melting point of the tripeptide is affected by the rate of heating. Two samples of the crystals melted at 190–192°. There was evolution of gas but no charring.

Constitution of the Tripeptide, Treatment with Nitrous Acid.—3.07 gm. of the recrystallized tripeptide were dissolved in 50 cc. of water. 2.1 gm. (3 equivalents) of sodium nitrite were added. The solution turned bright red. 6 cc. of 5 N sulfuric acid, which was equivalent to the nitrite, were added slowly with cooling. A large volume of gas was given off. The solution was placed in a vacuum to remove the last trace of nitrous acid. Concentrated hydrochloric acid was added so that the resulting volume of 100 cc. contained approximately 25 per cent of hydrochloric acid. The solution was refluxed overnight. The red color rapidly disappeared when hydrochloric acid was added; the solution was light yellow. After removal of the hydrochloric acid by evaporation to dryness in a vacuum, 100 cc. of water were added and cystine was precipitated with mercury sulfate. Mercury was removed from the filtrate, the sulfuric acid was removed with a measured weight of barium hydroxide. The barium sulfate was removed by filtration and 30 cc. of N sulfuric acid, equivalent to the barium were again added. The solution was concentrated to 25 cc. and 150 cc. of alcohol were added. Sodium sulfate crystallized out and was removed. The solution was filtered, the alcohol removed, and concentrated to a small volume which

was saturated with hydrochloric acid gas. No crystals of glutamic hydrochloride formed. A small amount of sodium chloride was present. The solution was again placed under a vacuum and, after removal of the hydrochloric acid, benzoyl chloride and sodium hydroxide were added as already described. From this, 1.05 gm. of hippuric acid were separated. The melting point was 188°.

Oxidation of the Tripeptide (8).—1 gm. of the recrystallized glutathione was dissolved in 10 cc. of water to which were added 4 cc. of concentrated ammonia, 5 mg. of ferrous sulfate, and 50 cc. of 5 per cent hydrogen peroxide (3, 4). The solution, was warmed to 70°. It was then just acidified with hydrochloric acid and oxygen was passed through the solution to force out the carbon dioxide which was passed through a standardized solution of barium hydroxide. 98 cc. of 0.1 N carbon dioxide were given off. This indicates the liberation of 1.5 equivalents of carbon dioxide. The solution was evaporated to dryness in a vacuum, redissolved in a small volume of water, and was extracted with ether. No organic acid was soluble in the ether. Hydrochloric acid was then added and the solution was refluxed for 20 hours. It was evaporated to a small volume and again extracted with ether. The organic acid in the ether weighed 235 mg. By carboxyl titration and formation of the silver salt this was identified as succinic acid. The solution was benzoylated but benzoyl glycine could not be separated. These results show that both the glutamic acid and the glycine were destroyed by oxidation. Oxalic acid was not present.

Solubility and Stability of the Tripeptide.—The solubility of the tripeptide depends on the purity of the material. In the presence of the products precipitated with mercury and subsequently liberated with hydrogen sulfide, the tripeptide is exceedingly soluble. It seems highly probable that practically all of the cysteine present is in a form of the tripeptide. As shown in Table I, however, the amount of crystalline material which can be separated does not exceed about a half of the total weight. When the crude crystals are dissolved in water, only about a half of the weight of the material can be recrystallized. But as the crystallization is continued, the tripeptide becomes less and less soluble. When pure, it is non-hydroscopic, and is soluble to the extent of about 1 part in 10 parts of water at 0°. It is easily soluble in warm

water. 3 gm. of the uncrystallized material which had been precipitated with absolute alcohol from the mother liquor of the crystals was boiled in 200 cc. of 0.1 N hydrochloric acid. The hydrochloric acid was removed by evaporation to dryness in a vacuum and the residue was redissolved in water and was precipitated with mercury sulfate. Mercury was removed from the filtrate which was then concentrated and treated with benzoyl chloride in the presence of sodium hydroxide. Benzoyl glycine could not be separated from the solution.

3 gm of the same material which were hydrolyzed in concentrated boiling hydrochloric acid for 18 hours gave a yield of 1.3 gm. of hippuric acid when treated in an entirely similar way. This is evidence that the glycine is not easily broken off from the tripeptide in boiling 0.1 N hydrochloric acid. Preliminary experiments have indicated that the glycine may be broken off from the tripeptide by enzymic action.

Throughout this work, we have been greatly assisted in the analyses of the solutions and in some of the analytical work by Daisy Simonsen and Dr. A. E. Osterberg.

SUMMARY.

Hopkins' method for the isolation of glutathione from yeast has been modified as follows:

1. The suspension of yeast is extracted with cold water in the presence of benzene. The cells are removed in a large centrifuge. The solution is precipitated with neutral lead acetate. The pH of the solution must be about 5.5. The lead precipitate is decomposed with sulfuric acid and some impurities are removed by raising the pH to 4.0 with barium hydroxide. The solution is made acid and is treated with phosphotungstic acid at 0°. The phosphotungstic acid is removed with barium and the glutathione is precipitated with mercury sulfate. The mercury precipitate is decomposed with hydrogen sulfide. Sulfuric acid is removed from the solution which is then concentrated to a small volume. On standing, the solution sets to a crystal mass and the crystals are washed with glacial acetic acid and absolute alcohol. They may be recrystallized from water.

2. This material is a tripeptide of glutamic acid, glycine, and cysteine. The glycine is attached to the carboxyl group of

glutamic acid, which is nearest to the amine group. Cysteine is attached to the other carboxyl group of the glutamic acid. The chemical reactions used in the determination of the structure of the tripeptide are given.

3. The material precipitated from the mother liquor of the crystals with absolute alcohol has very nearly the same percentage composition and glycine can be separated after hydrolysis from this material and from the crystalline tripeptide in about the same yield. It seems highly probable that practically all of the cysteine is present in the form of the tripeptide.

4. The formula suggested in this paper is based on three facts. First, succinic acid is obtained after hydrogen peroxide treatment only after hydrolysis. Second, glycine but no glutamic acid can be separated after treatment with nitrous acid. Third, neither glutamic acid nor glycine can be separated after oxidation with hydrogen peroxide. The fact last mentioned is negative in character; therefore, until more evidence positive in nature is obtained, the structure of the tripeptide must remain in doubt.

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AROMATIC ALDEHYDE DERIVATIVES OF PROTEINS, PEPTIDES, AND AMINO ACIDS.

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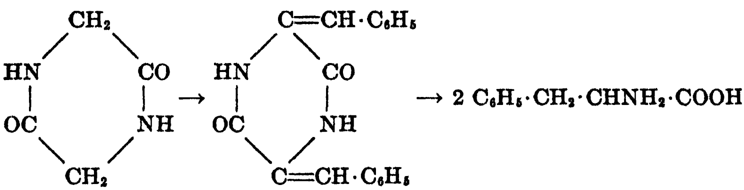
The following paper contains a preliminary account of some aromatic aldehyde derivatives of proteins and related substances which appear to be of a type hitherto undescribed. It may perhaps be permissible to indicate briefly the reasons for making the experiments which lead to the isolation of the new compounds even though the original purpose of the experiments signally failed. As is well known, considerable interest has been shown in recent years as to whether complex proteins contain diketopiperazine rings in addition to the ordinary straight chain amide linking of amino acids. It will also be recalled that Sasaki (1) has shown that aromatic aldehydes readily condense with the methylene groups of glycine anhydride (Formula I) with elimination of water, forming compounds (Formula II) which on reduction and hydrolysis give phenylalanine (Formula III) and its derivatives.



I. II. III.

Since most evidence as to the presence of diketopiperazine rings in proteins is of an indirect kind, it was thought possible that more direct information as to their presence might be obtained by condensing a glycine-rich protein such as gelatin directly with an aromatic aldehyde in the hope that on subsequent hydro-

lysis or other treatment evidence might be obtained of the presence of reactive methylene groups such as occur in glycine anhydride. Accordingly benzaldehyde was condensed with gelatin in the presence of sodium acetate and acetic anhydride. The reaction proceeded surprisingly easily, and with little trouble a characteristic derivative was isolated in good yield. It was found however that the reaction had taken an entirely unexpected course for the product was extraordinarily resistant to hydrolysis. Moreover it was soon found that other proteins which contain no glycine and hence could have no reactive methylene groups, such as occur only in glycine anhydride, furnished entirely analogous derivatives with aromatic aldehydes. The proteins used were chiefly casein, zein, egg albumin, and gelatin, while the aldehydes included benzaldehyde, salicylic aldehyde, chlorobenzaldehyde, various nitrobenzaldehydes, and *p*-dimethylaminobenzaldehyde. The condensation products were readily isolated, as described in the experimental section, first of all on account of their ready solubility in dilute sodium hydroxide followed by their precipitation with mineral acids, and secondly by their great solubility in alcohol and precipitation by ether from their alcoholic solutions. The substances in all cases were yellow to brown amorphous powders and could not be crystallized—consequently no guarantee as to their purity can be offered though their relative constancy of composition is certainly evidence as to their definite nature. They are acidic substances and dissolve readily in alkaline hydroxides but are precipitated from these solutions by carbon dioxide. Ammonia and pyridine dissolve them, while sodium carbonate only dissolves traces. The biuret reaction is completely negative and the only typical protein reaction that persists is their ability to couple with diazo compounds in alkaline solution to give brown or orange dyes. Millon's reagent and the tryptophane reaction with glyoxylic acid are atypical. They are extraordinarily resistant to hydrolysis, and prolonged boiling with concentrated alcoholic hydrochloric acid or 20 per cent sodium hydroxide leaves the bulk of the substance more or less unchanged. Their reaction toward potassium permanganate is significant. On adding dilute permanganate to a solution of a benzaldehyde-protein derivative dissolved in 0.2 N sodium hydroxide, there is no precipitation of manganese dioxide for a considerable time but

bright green manganate is immediately formed. At no time is benzaldehyde liberated but slow reduction of the manganate takes place on standing with production of some benzoic acid and acidic protein derivatives which are soluble in alcohol but precipitated by ether. It may be fairly concluded from the foregoing that no benzylidene groups linked to carbon are present in the compounds and that aldehyde condensation products of the type that might be formed from glycine-anhydride groups, if present in the protein molecule, are absent. The action of prolonged boiling with concentrated hydriodic acid and red phosphorus was also investigated. While decomposition was very incomplete, the products of the reaction were specially examined for the possible formation of benzylamine or some similar base, but none was found.

On comparing the elementary composition of various aldehyde derivatives with that of the proteins from which they were prepared, it became evident that the substances were not essentially condensation products formed with the elimination of water—the oxygen being furnished by the aldehyde—but that they could be as well or better represented as made up of x aldehyde + y protein, although of course their chemical stability precludes any possibility of their being simple addition compounds. The proportion of aldehyde to protein appears constant for each protein, but individual proteins show a marked difference in the amount of aldehyde with which they combine. For example gelatin combines with much more benzaldehyde than casein or zein. The proportion of aldehyde to protein was confirmed by using p-chlorobenzaldehyde and analyzing the purified product for halogen, while nitrogen estimation furnished a basis for estimating the amount of protein.

Table I gives a few typical analyses of these protein aldehyde compounds, together with the usually accepted values of the proteins themselves. In the lower half of the table some calculated values for products of the type x protein + y aldehyde are included for purposes of comparison.

Consideration of the analytical results in Table I shows that while a protein such as gelatin containing a high percentage of nitrogen (18.0) unites with considerably more aldehyde than zein or casein (15.7 to 16.1 per cent N), the proportion of aldehyde

to "monoamino nitrogen" is less than 1:1 but more than 1:2. A simple calculation will show that the molecular proportion of benzaldehyde and chlorobenzaldehyde to protein is about the same in the case of both gelatin and casein.

TABLE I.

Substance.	Carbon.	Hydrogen.	Nitrogen.	Chlorine.
Gelatin	49.4	6.8	18.0	
Gelatin-benzaldehyde	58.5–59.7	6.26–6.40	11.0	
Gelatin-chlorobenzaldehyde	53.5	5.40	9.20–9.66	11.6–12.6
Casein	53.3	7.05	15.7	
Casein-benzaldehyde	61.7–62.1	6.86–7.20	11.1–11.3	
Casein-chlorobenzaldehyde	55.0	5.85	9.5–10.2	8.3–8.72
Zein	55.2	7.26	16.1	
Zein-benzaldehyde	60.5–60.9	7.09–7.30	11.8	
Calculated values for:				
Gelatin 62 per cent. Benzaldehyde 38 per cent.	60.7	6.21	11.1	
Gelatin 54 per cent. Chlorobenzaldehyde 46 per cent.	54.1	5.21	9.72	11.5
Casein 70 per cent. Benzaldehyde 30 per cent.	61.1	6.63	11.0	
Casein 64 per cent. Chlorobenzaldehyde 36 per cent.	55.5	5.80	10.0	9.10
Zein 73 per cent. Benzaldehyde 27 per cent.	61.5	6.83	11.8	

In an endeavor to learn more of the nature of these protein aldehyde compounds some aldehyde derivatives of amino acids and dipeptides have been prepared. The simplest derivative from glycine has already been described (2), and analogous compounds from alanine and leucine are included in this paper. These compounds all appear to be of the type $(R \cdot CH \cdot (COOH)N{=}CH \cdot C_6H_5)_n$. The dipeptides on the other hand furnish yellow amor-

phous substances which approximate in composition compounds derived from 2 molecules of benzaldehyde with elimination of 1 molecule of water. These substances, prepared from glycylglycine, glycylleucine, and alanylglycine show considerable superficial resemblance to the protein derivatives. Glycinamide gives an analogous compound. They are acidic substances, soluble in alcohol, precipitable by ether from alcohol, and show the same reaction toward potassium permanganate in dilute alkaline solution. A great variety of formulæ may be constructed as possibly representing these compounds, but in the absence of convincing experimental evidence it seems hardly worth while discussing them in detail. Possibly they contain the isoxazole ring. It would appear significant that in sharp contrast to the dipeptides a typical diketopiperazine, namely leucine anhydride, gave only a loose aldehyde condensation product which was readily resolved into aldehyde and unchanged anhydride on working up the products of the reaction.

If a protein such as gelatin or casein were constructed entirely of amino acids united by peptide (amide) linkages, the results with the dipeptide would lead one to expect a significantly higher proportion of aldehyde to protein in the previously described compounds than is actually found to be the case. The explanation of the experimental results may of course be interpreted in many ways. First of all there is the question of diketopiperazine groupings which would not bind aldehyde in stable combination, secondly steric hindrance might well be a factor, while as a third suggestion it is possible that iminazole rings are formed in which two (NH) groups from adjacent amino acid groups condense with 1 molecule of aldehyde in a manner somewhat analogous to the formation of amarine and lophine from benzamide. At the present time detailed speculation seems unprofitable and must await further experimental results. It may be mentioned however that "biuret base," the ethyl ester of triglycylglycine, gave a compound indicative of three rather than four aldehyde groups. Further experiments with the higher polypeptides is required. It may be noted that cinnamic aldehyde and related aldehydes also give condensation products with proteins resembling their benzaldehyde analogues. The biological properties of the protein aldehyde compounds are being investigated by my friend Mr. A. Wormall, of the University of Leeds.

EXPERIMENTAL.

Since the preparative methods used for the various proteins and aromatic aldehydes were essentially the same, the description of a single typical preparation will suffice.

Gelatin-Benzaldehyde.—Finely powdered gelatin (10 gm.), anhydrous sodium acetate (10 gm.), benzaldehyde (10 cc.), and acetic anhydride (30 cc.) were heated for an hour on the water bath, and then 20 cc. more acetic anhydride were added to the jelly-like mass which was then heated for 4 hours in an oil bath at 130–135°. Solution was then complete. Steam was next blown through the mixture to remove most of the unchanged aldehyde. Insoluble material only began to separate toward the end of the distillation. The contents of the flask were then cooled and the solution made acid to Congo red with dilute sulfuric acid. The sticky mass was washed with cold water and then dissolved in 100 cc. of normal sodium hydroxide with gentle warming on the water bath. The dark brown solution was next chilled and filtered from a little insoluble material. The filtrate was then precipitated with hydrochloric acid. The precipitate was washed with cold water and gave 8 gm. of air-dried material. It was dissolved in a minimum amount of methyl alcohol (about 15 cc.), filtered, and then precipitated with excess of anhydrous ether. The solution in alcohol and precipitation with ether was repeated. The product separates in lumpy brown masses which are washed with ether and dried. The yield is 4.5 to 6.0 gm., and the other proteins and aldehydes mentioned in the earlier part of the paper give about the same yield. The properties and composition of the substances have already been described in the introduction and need not be repeated.

Alanine and Benzaldehyde.—Alanine (2 gm.), sodium acetate (2 gm.), benzaldehyde (5 cc.), and acetic anhydride (10 cc.) were heated at 100° for 5 hours. Steam was then blown through the mixture and the residue acidified with dilute sulfuric acid. It is of interest to note that the crude precipitate contains a considerable amount of cinnamic acid which would not be formed at the relatively low temperature employed unless alanine were present. It appears therefore that the cinnamic acid synthesis is catalyzed by alanine and other amino acids in much the same way that

other synthetic reactions have been shown to be accelerated by amino acids (3).

The crude product was dissolved in hot 0.5 N sodium hydroxide, the solution cooled, filtered, and reprecipitated by addition of hydrochloric acid. After drying the precipitate, it was dissolved in a little alcohol and precipitated by petroleum ether. It was finally dissolved in a minimum of absolute alcohol and precipitated with dry ether. The product is a light yellow powder which begins to soften above 175° and melts indefinitely between 185–190°. For analysis the substance was dried *in vacuo* at 50°.

Analysis.

$C_{10}H_{11}O_2N$.	Calculated.	C 67 8, H 6.21, N 7.90.
	Found.	" 67.9, " 6.33, " 7.82.

Dipeptides and Benzaldehyde.—The dipeptides, glycylglycine, glycylleucine, and alanylleucine, were prepared according to Fischer's methods. In each case 2 gm. of the peptide were heated for 3 hours at 120° with sodium acetate (2 gm.), benzaldehyde (5 cc.), and acetic anhydride (10 gm.). The products were worked up exactly as described for the alanine derivatives. In each case some cinnamic acid was formed. The products were light yellow amorphous powders readily soluble in alcohol, insoluble in ether and petroleum, and decomposed indefinitely above 200°.

Analyses.

$C_{18}H_{18}O_4N_2$ (glycylglycine derivative).

Calculated.	C 66.3, H 5.5, N 8.6.
Found.	" 67.1, " 5 6, " 8.21.

$C_{22}H_{26}O_4N_2$ (glycylleucine derivative).

Calculated.	C 69.1, H 6.8, N 7.33.
Found.	" 69.4, " 6.8, " 7.30.

$C_{23}H_{28}N_2O_4$ (alanylleucine derivative).

Calculated.	C 69.7, H 7.07, N 7.07.
Found.	" 69.2, " 7.15, " 7.40.

Leucinimide and Benzaldehyde.—2 gm. of leucinimide were treated in the same way as the preceding dipeptides. When the reaction was carried out at 130° almost no alkali-soluble product was obtained except cinnamic acid. The experiment was therefore repeated at the lower temperature of 100° for 6 hours. The whole of the leucinimide went into solution. On steam distilla-

tion a slightly yellow horn-like mass was left. On boiling this with normal sodium hydroxide for a few moments, it decomposed and benzaldehyde was liberated. The solution was cooled, when over a gm. of unchanged leucinimide, m.p. 270°, separated out. The alkaline filtrate gave only a negligible trace of precipitate on acidifying and most of this was benzoic acid.

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THE MECHANISM OF EPINEPHRINE ACTION.

IV. THE INFLUENCE OF EPINEPHRINE ON LACTIC ACID PRODUCTION AND BLOOD SUGAR UTILIZATION.

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It has been shown in a previous paper that subcutaneous injections of epinephrine lead to an increase in blood lactic acid of rabbits and cats (1). The effect was more pronounced in the former species than in the latter. This increase in blood lactic acid after epinephrine injection was also observed in men (2) and rats (3) and it seems likely therefore that it occurs in all mammals. Other experiments made it seem probable that the lactic acid which was found in increased amounts in the blood had its source in muscle glycogen, since the latter was found to diminish after epinephrine injections (4, 5). In the rat it is possible to analyze the body *in toto* for glycogen and not merely corresponding muscles before and after injection. Non-glycosuric doses of epinephrine caused a disappearance of muscle glycogen in fasting rats in which the glycogen remained practically constant when no injection was given (5). The animals were killed 3 hours after the injection while active absorption of epinephrine from the subcutaneous tissue was still going on. In rats in the postabsorptive state, muscle glycogen disappeared nearly twice as fast after non-glycosuric doses of epinephrine as in uninjected controls or after insulin injections (4). Several other authors, who observed a decrease in muscle glycogen were cited in these two papers. More recently Chaikoff and Weber (6) state that they observed a decrease in muscle glycogen in the standard white rat after epinephrine. Geiger and Schmidt (7) observed mobilization of the glycogen depots of the muscles in phlorhizinized dogs, and they were able to account in this way for the extra sugar appearing in the urine. Blatherwick and Sahyun (8) noted a decrease in muscle glycogen

in rabbits, and Sahyun and Alsberg (9) made use of epinephrine to free the muscles of glycogen. Eadie (10) concludes that 1½ hours after administration of epinephrine to cats under amytal anesthesia the glycogen content of the muscles is unaltered, but this contention is not borne out by the experiments. This author published two control experiments and two experiments with epinephrine. In the former the muscle glycogen rose in one case from 0.95 to 0.99 per cent and in the other it fell from 1.09 to 0.97 per cent. After epinephrine injection the muscle glycogen of one animal diminished from 0.93 to 0.9 per cent, which is a negative result, but in the other it fell from 1.3 to 1.0 per cent. The author, who determined glycogen in only 10 gm. of muscle, does not seem to realize what this means when the whole muscle mass is taken into consideration. According to Best *et al.* (11) the muscles constitute 50 per cent of the body weight of cats. Since the animal weighed 2.93 kilos, the difference in muscle glycogen of 0.3 per cent must be multiplied by 14.65. In other words, in this experiment with epinephrine, when taken at its face value, muscle glycogen disappeared to the extent of 4.4 gm. During the same time only 0.8 to 0.9 gm. of glycogen disappeared from the liver. Hence, one is justified in concluding that the action of epinephrine on muscle glycogen is far more important from a quantitative standpoint than the action on liver glycogen, which is the opposite conclusion from that reached by Eadie. In view of this, it seems remarkable that the author did not continue his observations before publishing his results. The point is, of course, that in 1½ hours epinephrine might mobilize a considerable quantity of muscle glycogen, as shown in the above calculation, without appreciably affecting the percentage of glycogen in one individual muscle or, indeed, exceeding the considerable error involved in this type of experiment. When blood lactic acid is determined under the conditions obtaining in Eadie's experiments, definite evidence is obtained that epinephrine acts on muscle glycogen.

In the present experiments the relation between the blood sugar and lactic acid curves after epinephrine injection was studied in detail and lactic acid was also determined in arterial and venous blood of the leg. In this way it could be shown that mobilization of muscle glycogen is a constant as well as an early effect of epinephrine injections. Since insulin in suitable doses prevents the rise

in blood sugar after epinephrine injection, it seemed of interest to see whether insulin would have the same effect on blood lactic acid. Insulin alone does not lead to an appreciable increase in blood lactic acid, provided the blood sugar remains above the level at which hypoglycemic symptoms occur (1, 12). It was found that insulin has very little effect on the increase of blood lactic acid after epinephrine injection, a point which will be discussed later.

EXPERIMENTAL.

Male rabbits of a quiet disposition were kept for some time in the laboratory on a diet of green vegetables, carrots, oats, and table scraps, to insure their good health. They were fasted for

TABLE I

Control Experiment.

Time	Blood sugar.	Blood lactic acid	Remarks
	mg per 100 cc	*mg per 100 cc*	
9 40 a m.	120	7 8	Rabbit A, weight 2300 gm No injections.
10 40 "	117	10 5	
11 40 "	110	14 1	
12 40 p m	112	11 1	
1 40 "	111	10 7	

24 hours previous to the experiments. Dilatation of the marginal ear vein was produced by rubbing with xylene and a clean cut was made by means of a razor. The blood flowed freely, and the collection was effected in a short time and with a minimum of stasis. The bleeding was stopped by application of some cotton and a short compression. For the next bleedings it was merely necessary to tear away the cotton and rub the incision with xylene. The rabbits were sitting in boxes which afforded little opportunity for movement. While the blood was being taken, which lasted only 1 minute, the animals were perfectly quiet and showed no signs of excitement. Generally two samples of blood were removed before either epinephrine alone or combined with insulin was injected. The respective doses of the two hormones are given in Table II. No signs of discomfort or twitching of muscles were

TABLE II.

Blood Sugar and Lactic Acid Curve after Epinephrine and Insulin Plus Epinephrine.

The results are expressed in mg. per 100 cc. of blood.

	Blood sugar.	Blood lactic acid.	Blood sugar.	Blood lactic acid.	Remarks.
Before.	122		123	15 7	Rabbit 1, weight 2400 gm.
"	129	13 1	138	16 9	
0.5 mg. epinephrine.			40 units insulin, 0.5 mg. epinephrine.		
1 hr.	305	78 1	124	71.6	
2 hrs.	312	73 6	98	67 2	
3 "	254	66 4	80	52 7	
4 "	198	67.9	65	48 2	
Before.	121	7 4	115	13 5	Rabbit 2, weight 2300 gm.
"	142	8.9	130	15 1	
0 5 mg. epinephrine.			20 units insulin, 0.5 mg. epinephrine.		
1 hr.	332	79 1	194	69 5	
2 hrs.	399	50 6	169	55 8	
3 "	352	33 9	106	37.8	
4 "	244	23 4	80	31 2	
Before.	120		112	14 8	Rabbit 3, weight 2300 gm. Convulsions 10 min. after last blood sampling.
"	121	14 6	20 units insulin, 0.5 mg. epinephrine.		
0.5 mg. epinephrine.					
1 hr.	320	62 5	93	54 3	
2 hrs.	350	52 4	62	43 7	
3 "	305	44 1	46	45 1	
4 "	180	29 5			
Before.	116	11 1	132	7 2	Rabbit 4, weight 1700 gm.
"	131	10 6	126	8 3	
0.5 mg. epinephrine.			15 units insulin, 0.5 mg. epinephrine.		
1 hr.	381	75.9	222	61 4	
2 hrs.	417	73 7	209	40.3	
3 "	416	51 2	146	27.4	
4 "	321	28 3	105	23 0	

observed in the rabbits after epinephrine injection. Blood samples were removed every hour for 4 hours and a total quantity of 9 to 10 cc. of blood was withdrawn during that time. A con-

trol experiment was performed in order to show that the removal of such quantities of blood and the handling of the rabbits are without influence on sugar and lactic acid content of the blood (Table I).

Blood sugar was determined by means of the Hagedorn and Jensen method (13) in the same Folin-Wu filtrate in which the lactic acid determination was carried out. For lactic acid the method of Friedemann and Kendall (14) was used with excellent

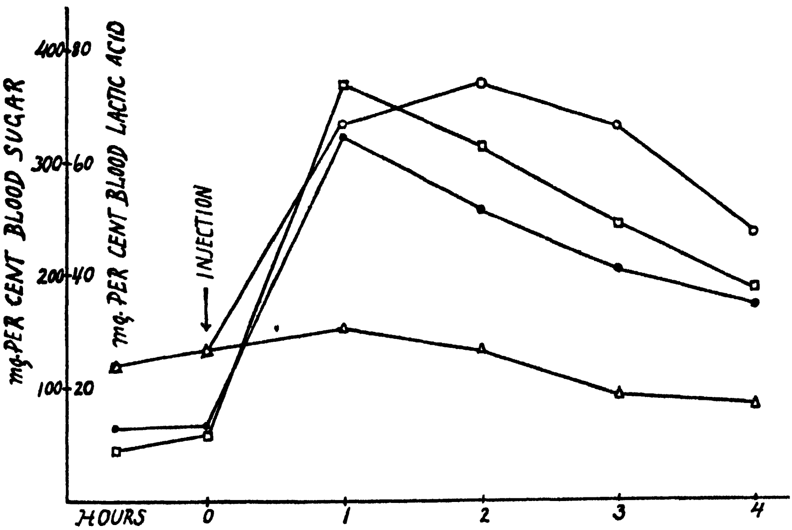

FIG. 1. Graphic representation of the average values calculated from Table II. ○—— ○ represents blood sugar after epinephrine; □—— □, blood lactic acid after epinephrine; △—— △, blood sugar after epinephrine plus insulin; ●—— ●, blood lactic acid after epinephrine plus insulin.

success. The recoveries of lactic acid in pure solution and after addition to blood were very satisfactory. Frequent determinations of the reagent blank were found essential in an effort to determine as nearly as possible true lactic acid values.

Two experiments, one with epinephrine alone and one with insulin plus epinephrine, both injected subcutaneously in different skin regions, were performed on the same rabbit. An interval of 8 to 12 days was allowed between two experiments and care was

taken to alternate the order in which the two experiments were made in the different rabbits. The results obtained are shown in Table II. A compound curve of the average values of Table II was drawn in Fig. 1. By way of comparison, glucose was injected intravenously into two normal rabbits, the aim being to determine at what rate glucose must be supplied in order to obtain the same blood sugar curve as after epinephrine injection (Table III). This rate was found to be greater than 2.5 gm. of glucose per kilo per

TABLE III.

Blood Sugar and Lactic Acid Curve after Glucose Injection.

	Blood sugar.	Blood lactic acid.	Remarks.
hrs.	*mg. per 100 cc.*	*mg per 100 cc.*	
Before injection.	130	20 1	Rabbit B, weight 1800 gm. 1.27 gm. glucose intravenously every 20 min. for 3 hrs.; total injected 11.43 gm. (2.1 gm. glucose per kilo per hr.).
After beginning injection.			
1	270	23 0	
2	286	27 6	
3	223	22 1	
4	139	18 8	
Before injection.	125	18 2	Rabbit C, weight 2300 gm. 1.92 gm. glucose intravenously every 20 min for 3 hrs.; total injected 17.28 gm. (2.5 gm. glucose per kilo per hr.).
After beginning injection.			
1	297		
2	312	22 6	
3	306		
4	153	17 9	

hour. The experiments in Table III also show that hyperglycemia as such does not lead to marked changes in blood lactic acid.

Another group of rabbits was used in experiments which were designed to show the site of lactic acid formation after epinephrine injection. For this purpose one femoral vein and carotid artery were exposed under amytal anesthesia (60 to 70 mg. per kilo intraperitoneally) with as little trauma as possible. Hinsey and Davenport (15) have shown that amytal does not cause a decrease of muscle glycogen. Anesthesia was used to insure complete muscular relaxation. The rabbits were not tied down during the experi-

TABLE IV.

Comparison of Arterial and Venous Blood Sugar and Lactic Acid after Epinephrine.

The animals were under amytal anesthesia.
The results are expressed in mg. per 100 cc. of blood.

	Blood sugar.			Blood lactic acid.			Remarks.
	Artery.	Vein.	Difference (V − A)	Artery.	Vein	Difference (V − A).	
Before injection.	149	146	−3	14 3	12 5	−1 8	Rabbit 5, weight 3000
After injection.							gm. 1 mg. epineph-
1 hr.	320	318	−2	41 9	47 1	+5 2	rine subcutane-
2 hrs.	350	351	+1	33 2	39 9	+6 7	ously.
3 "	327	324	−3	27 5	29 1	+1 6	
Before injection	148	144	−4	24 4	24 0	−0 4	Rabbit 6, weight 2800
After injection.							gm. 1 mg epineph-
30 min.	225	220	−5	57 0	70 7	+13 7	rine subcutane-
1 hr.	306	300	−6	76 6	86 9	+10 3	ously.
2 hrs.	355	353	−2	50 1	53 7	+3 6	
Before injection	131	128	−3	13 7	13 9	+0 2	Rabbit 7, weight 2400
After injection.							gm. 0.5 mg. epi-
30 min.	182	178	−4	24 8	29 4	+4 6	nephrine subcuta-
1 hr.	225	220	−5	41 4	55 3	+13 9	neously.
2 hrs	268	255	−13	44 8	55 6	+10 8	
3 "	283	275	−8	56 1	58 6	+2 5	
Before injection.	145	135	−10	17 8	19 9	+2 1	Rabbit 8, weight 3100
After injection							gm. 1 mg. epineph-
30 min.	228	215	−13	25 5	33 7	+8 2	rine subcutane-
1 hr.	258	253	−5	29 7	45 8	+16 1	ously.
2 hrs.	303	294	−9	51 9	70 3	+18 4	
3 "	330	315	−15	52 2	59 8	+7 6	
Before injection.	140	137	−3	16 9	15 4	−1 5	Rabbit 9, weight 2500
After injection.							gm. 1.0 mg. epin-
30 min.	245	240	−5	21 1	41 3	+20 2	ephrine subcutane-
1 hr.	298	295	−3	51 1	55 6	+4 5	ously.
2 hrs.	368	360	−8	65 2	75 9	+10 7	
3 "	355	352	−3	57 3	74 3	+17 0	

ment. Blood was drawn from the vein without interfering in any way with the blood flow, and within 1 minute a sample of blood was taken from the carotid artery. Difficulty from bleeding was not encountered and all blood samples were removed from the same

TABLE V.

Comparison of Arterial and Venous Blood Sugar and Lactic Acid during Glucose Injection at Constant Rate.

The animals were under amytal anesthesia.

The results are expressed in mg. per 100 cc. blood.

	Blood sugar.			Blood lactic acid.			Remarks.
	Artery.	Vein.	Difference (V − A).	Artery.	Vein.	Difference (V − A).	
hrs.							
Before injection.	129	129	±0	10.4	11.9	+1.5	Rabbit D, weight 2700 gm. 1.66 gm. glucose per kilo per hr. for 3 hrs.
After beginning injection.							
1	349	330	−19	19.8	20.2	+0.4	
2	306	289	−17	23.9	23.1	−0.8	
3	285	270	−15	24.4	20.4	−4.0	
Before injection.	163	160	−3	14.8	11.3	−3.5	Rabbit E, weight 1900 gm. 1.45 gm. glucose per kilo per hr. for 3 hrs.
After beginning injection.							
1	328	315	−13	16.3	10.4	−5.9	
2	330	312	−18	17.7	15.7	−2.0	
3	358	344	−14	26.7	21.1	−5.6	
Before injection.	145	144	−1				Rabbit F, weight 2400 gm. 1.68 gm. glucose per kilo per hr. for 3 hrs.
After beginning injection.							
1	322	286	−36				
2	351	324	−27				
3	299	280	−19				

vein. All animals survived the experiments indefinitely. Whereas before the injection of epinephrine venous blood coming from the leg contained less lactic acid than arterial blood, this was reversed after the injection. This result is shown in Table IV. It is of importance to note that the blood lactic acid increased

after epinephrine in spite of the fact that the animals were at absolute rest on account of the anesthetic. Three control experiments were performed in which hyperglycemia was produced by means of an injection of glucose (Table V). In this case the venous blood contained less lactic acid than arterial blood, and the blood lactic acid did not rise as during epinephrine hyperglycemia. Special attention is called to the arteriovenous blood sugar differences. When epinephrine is injected, the differences remain small

TABLE VI.

Influence of Epinephrine on Blood Lactic Acid of Cat.

Time.	Blood sugar.	Blood lactic acid.	Remarks.
	mg per 100 cc.	*mg. per 100 cc.*	
10 25 a.m.	111	8.6	July 17, 1929. Weight 3200 gm.
10 50 "	105	10.7	
10.53 "			0.6 mg. epinephrine subcutaneously.
11.53 "	232	40.6	Animal restless.
12.53 p.m.	294	31.8	" "
1 53 "	320	29.1	
2.53 "	300	15 5	
			July 26, 1929.
9 40 a.m.			180 mg. amytal subcutaneously.
10 40 "			45 " " "
12 20 p.m.			45 " " intraperitoneally.
12 42 "	91	11.2	
12.45 "			3 " epinephrine subcutaneously.
1.30 "	272	38.3	
2 15 "	260	52 2	
3 15 "	271	42 7	

in spite of the marked hyperglycemia. In contrast to this, it can be seen in the control experiments in Table V that the muscles take up more sugar when hyperglycemia is produced by an injection of glucose.

In Table VI two experiments on a cat are recorded. In a preliminary test 0.2 mg. of epinephrine per kilo was injected into the unnarcotized animal. This was followed by a marked rise in blood sugar and lactic acid, though the dose was 5 times smaller than the one used by Eadie. The curves for both blood sugar and

lactic acid resemble those obtained on rabbits with the exception that all values, especially those for lactic acid, tend to be lower. In the second test exactly the same conditions as in the experiments of Eadie were established, because it seemed advisable to see what effect the prolonged anesthesia has on lactic acid production in muscle. The cat was given 60 mg. of amytal per kilo subcutaneously, followed 30 minutes later by 15 mg. This did not prove to be sufficient anesthetic, because the animal showed violent shivering, and another 15 mg. per kilo was therefore given intraperitoneally. After 3 hours of anesthesia a blood sample was removed and the dose of epinephrine that Eadie used (1 mg. per kilo) was injected subcutaneously. A marked increase in blood lactic acid resulted, which shows that a breakdown of muscle glycogen occurred in this experiment.

DISCUSSION OF RESULTS.

The previous observation that subcutaneous injections of epinephrine produce a marked rise in the lactic acid content of the blood is confirmed and amplified. The increase occurs shortly after the injection and reaches its maximum in 1 hour. In the next 4 to 5 hours the blood lactic acid gradually returns to the initial level. This is the effect to be observed in rabbits following the injection of 0.2 to 0.26 mg. of epinephrine per kilo (Table II, and Fig. 1). After large doses the increase in blood lactic acid is greater, the peak of the lactic acid curve occurs after 2 hours or later, and the return to normal is more prolonged.

The shape of the lactic acid curve depends on the rate of production of lactic acid and the rate of removal of lactic acid from the blood. Evidence is offered in the present paper that the lactic acid production occurs in muscle and that it persists for several hours; that is, as long as epinephrine is being absorbed into the blood stream. By comparing the lactic acid content of arterial and venous blood it was found that blood drawn at various time intervals from the femoral vein following epinephrine injections contained decidedly more lactic acid than arterial blood, while before the injection venous blood from the leg contained the same amount or less lactic acid than arterial blood (Table IV). The average differences in Table IV were as follows: Before the injection there was 0.3 mg. less lactic acid in venous than in arterial

blood; 30, 60, 120, and 180 minutes after the injection there were 11.7, 10.4, 10.0, and 7.1 mg. more lactic acid in venous than in arterial blood. When hyperglycemia was produced by glucose injection, there occurred only a slight rise in blood lactic acid and venous blood contained less lactic acid than arterial blood (Table V). As regards removal of lactic acid from the blood, it was shown in a previous paper (5) that a large part of the lactic acid derived from muscle glycogen is converted into liver glycogen and it was pointed out that epinephrine, since it is responsible for this transfer of muscle glycogen to liver glycogen, makes the former available as blood sugar.

The blood sugar curve after epinephrine injection formed a more or less distinct plateau, since there was not much difference in the average blood sugar values of the 1, 2, and 3 hour periods after the injection (Fig. 1). After 4 hours the blood sugar was still markedly elevated. A point to be considered carefully is the quantitative aspect of a hyperglycemia of such magnitude and duration as occurs after epinephrine injections. It is generally accepted that liver glycogen is the immediate source of blood sugar, and it is evident therefore that mobilization of liver glycogen must be involved in the production of epinephrine hyperglycemia.[1] However, it can easily be shown that such quantities of glycogen as are usually present in the liver, even if they would be mobilized completely, could only lead to a hyperglycemia of short duration, provided the tissues retain their normal ability to utilize sugar. In order to arrive at a quantitative estimate it is merely necessary to determine how much glucose must be injected intravenously into a normal rabbit in order to produce a blood sugar curve similar to that after epinephrine injection. If this is done, one finds that more than 2.5 gm. of glucose per kilo per hour must be administered (Table III). From this it can be calculated that the liver of a rabbit of 2 kilos, containing 5 per cent glycogen (about 4 gm.), could supply such quantities of sugar not even for 1 hour, while the epinephrine hyperglycemia lasts for 5 hours or longer and leads to considerable sugar excretion

[1] This has been expressed clearly on several occasions and also in a summarizing article (16). The fact that blood sugar is of hepatic origin does not necessarily mean that the liver and no other organ is responsible for the epinephrine hyperglycemia.

in the urine. What is, then, the mechanism of epinephrine hyperglycemia? Two possibilities have to be considered. One is that the rabbit receiving epinephrine utilizes less blood sugar than the rabbit receiving an intravenous injection of 2.5 gm. of glucose per kilo per hour. In this case the supply of blood sugar by the liver could last longer and the explanation would be that a low rate of mobilization of liver glycogen leads to hyperglycemia because epinephrine decreases the utilization of blood sugar in the tissues. The other possibility is that liver glycogen is being mobilized at a high rate (above 2.5 gm. per kilo per hour), but that new liver glycogen is being formed as fast as it disappears. In this case there would be no need for the assumption of a decreased utilization of blood sugar in the tissues. Finally a combination of both possibilities might occur.

A new formation of liver glycogen from blood lactic acid was found in previous experiments (5) but the rate of this process is too low to enable the liver to produce the required large amounts of blood sugar. On the other hand, a lowered utilization of blood sugar in the peripheral tissues was clearly observable in glucose-fed and epinephrine-injected rats (17). This low utilization was regarded as the essential factor necessary to account for the long drawn out epinephrine hyperglycemia. Obviously, some mobilization of liver glycogen must occur in order to raise the blood sugar level but the rate of this mobilization is not excessive after small doses of epinephrine, as was shown by the fact that in the rat under various conditions new formation of liver glycogen from blood lactic acid overbalanced the loss incurred by glycogen mobilization. A low utilization of blood sugar is also shown in the present experiments. The normal difference in arterial and venous blood sugar of unanesthetized rabbits was the subject of a previous investigation and was found to be 7 mg. in favor of arterial blood as an average of 60 observations (18). In the present experiments on rabbits under amytal (Table IV) the normal average difference was smaller (5 mg.). During epinephrine hyperglycemia the arteriovenous difference was hardly increased, and it can be stated quite definitely that the peripheral tissues did not respond to the hyperglycemia with increased withdrawal of sugar from the blood. Thus 30, 60, 120, and 180 minutes after the injection, the venous blood of the leg contained on an average

7, 4, 6, and 7 mg. less glucose than arterial blood. In contrast to this, average differences of 23, 21, and 16 were found when glucose was injected intravenously for 3 hours at a constant rate into rabbits under amytal anesthesia (Table V). Amytal lowers the glucose tolerance and it is therefore of importance that a similar result was obtained in unanesthetized rabbits in which a much milder hyperglycemia was produced by feeding glucose by mouth (18). In these experiments average differences of 20.3, 16.5, 17.2, and 11.8 mg. were found for the time intervals indicated. When insulin was injected along with the glucose feeding, the average differences between arterial and venous blood sugar were 25.7, 22.4, 25.0, and 19.0 mg. (18). Insulin is thus seen to accelerate the disappearance of sugar from the blood, and it becomes clear how insulin antagonizes epinephrine hyperglycemia and *vice versa.* These two hormones are mutually antagonistic mainly because of their divergent effect on blood sugar utilization.[2]

[2] Cannon believes that utilization of blood sugar in muscle is facilitated during epinephrine hyperglycemia and he is therefore opposed to our experimental evidence to the contrary; namely, that epinephrine decreases utilization of blood sugar. His statement (19) that emotional excitement can raise blood sugar 30 per cent and more in a few minutes and his objection that "the hyperglycemia comes too soon and is too clearly of hepatic origin to be ascribed to failure of use of glucose by peripheral tissues" are not to the point for two reasons. In the first place we explained the *persistence* of epinephrine hyperglycemia by a decreased utilization of blood sugar and not the initial rise in blood sugar. The latter has not yet been subjected to a thorough quantitative analysis. Secondly, we have repeatedly emphasized that blood sugar is of hepatic origin and a quotation (4) from one of our papers makes our standpoint clear; namely, " . . . that other factors play a rôle in the production of hyperglycemia besides mobilization of liver glycogen." Cannon also offers as evidence against our view the observation that "dogs exhausted by running can be made to continue (*i.e. using sugar in their muscles*) and will put forth from 17 to 44 per cent additional energy if they are given subcutaneously small doses of adrenin." (The italics are ours.) However, Cannon offers no experimental evidence that the additional muscular work was performed at the expense of blood sugar and he also states in another paper (20) that "it is clear that adrenalin has effects on muscular fatigue quite apart from mobilizing sugar in the blood or improving circulation." It was shown experimentally that under certain conditions epinephrine enables the muscles to utilize a greater proportion of their glycogen (4). The mobilizing action of epinephrine on muscle glycogen is also connected with the increase in blood lactic acid. Campos,

In the experiments with simultaneous administration of insulin and epinephrine (Table II), the dose of the latter was so chosen that the epinephrine hyperglycemia was either slight or absent or there was even a hypoglycemia. In spite of this marked effect on blood sugar, insulin had only a slight effect on blood lactic acid. Thus insulin did not prevent the marked rise in the 1st hour nor did it cause a more rapid disappearance of lactic acid in the following hours. With the exception of slightly lower values, the lactic acid curve in the experiments with insulin plus epinephrine is the exact duplicate of the curve obtained in the experiments with epinephrine alone (Fig. 1). This result is of interest in two respects. In the first place insulin can have no or very little effect on lactic acid production itself; in other words, it does not prevent the breakdown of muscle glycogen which is characteristic for epinephrine. Secondly, insulin hardly accelerates the removal of lactic acid from the blood. As has been mentioned before, the lactic acid accumulating in the blood as the result of epinephrine injections is partly removed by the liver where it is converted into glycogen. Insulin seems to be unable to accelerate this process. Little is known concerning the disposal of blood lactic acid in muscle under normal conditions. It seems clear, however, that insulin can have no marked effect on any possible oxidation of blood lactic acid in muscle or on its conversion into muscle glycogen. This seems remarkable since insulin is known to accelerate

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Cannon, *et al.* (20) confirm the increase in blood lactic acid after epinephrine injections but Cannon (19) is unwilling to accept the fact that mobilization of muscle glycogen occurs and is responsible for the increase in blood lactic acid, because he fails to see any advantage arising to the organism from such a mobilization. The possibility must at least be taken into consideration that the effectiveness of epinephrine in muscle fatigue may be due to its action on muscle glycogen. Finally, Cannon states that we "do not hint at the nature of the compensatory process" that leads to the disappearance of liver glycogen after insulin injections, "though we report low blood sugar levels which would set in action the sympathicoadrenal apparatus." Since we have shown that insulin injection causes a disappearance of liver glycogen in adrenalectomized animals (21), the adrenal apparatus cannot be responsible for this mobilization and we have more than hinted at the fact that this experimental evidence has not been disproved by Cannon though it is disregarded by him. The objections raised by Cannon in regard to the dose of epinephrine used in our experiments have been answered elsewhere (22).

storage and oxidation of glucose in muscle. Indeed this is the mechanism by which insulin prevents the epinephrine hyperglycemia. If insulin had the same effect on blood lactic acid as on glucose, it would also prevent the rise in blood lactic acid after epinephrine. The fact that insulin has such a small effect on the disposal of lactic acid casts some doubt on the assumption that lactic acid is an intermediary of glucose oxidation.

A discussion of the question of dosage will be presented in a later paper. It may be stated here that the minimal rate of intravenous infusion of epinephrine into unnarcotized rabbits which causes a perceptible rise in blood sugar also causes an increase in blood lactic acid. This minimal effective rate of infusion was found to be 0.00005 mg. of epinephrine per kilo per minute. The larger doses used in the present paper merely serve to illustrate better the changes that are taking place to a lesser extent with smaller doses.

SUMMARY AND CONCLUSIONS.

1. Subcutaneous injections of 0.2 mg. of epinephrine per kilo into rabbits are followed by marked changes in blood lactic acid. Starting from an average resting value of 11 mg. per cent, the lactic acid reaches a maximum value of 74 mg. per cent in 1 hour and gradually returns to the initial level in the next 5 hours. The peak of the blood sugar curve is reached in 2 hours and the hyperglycemia persists for about the same length of time as the increase in the blood lactic acid.

2. When insulin is given along with epinephrine, the rise in blood sugar can be suppressed completely, or, with smaller doses, markedly inhibited. In either case insulin has very little effect on the lactic acid curve. On an average, the values are 14 per cent lower than those obtained when epinephrine alone is injected; otherwise the two curves have a parallel course.

3. By means of lactic acid determinations in arterial and venous blood it was found that the peripheral tissues (muscles) are the source of the increase in blood lactic acid. Under resting conditions the average arteriovenous difference was 0.3 mg., while ½, 1, 2, and 3 hours after the epinephrine injection the lactic acid in venous blood was 11.7, 10.4, 10.0, and 7.1 mg. per cent higher than in arterial blood.

4. The arteriovenous blood sugar difference remained at the

resting value of 5 to 7 mg. per cent throughout the course of the epinephrine hyperglycemia. When glucose was administered the difference increased threefold. This indicates that less sugar passes from the blood to the muscles during epinephrine hyperglycemia than during the hyperglycemia produced by glucose administration.

5. More than 2.5 gm. of glucose per kilo per hour must be injected intravenously into normal rabbits in order to produce a blood sugar curve similar to that after epinephrine injection. Such quantities of sugar are not at the disposal of the liver. Since during epinephrine action the peripheral tissues do not respond with increased blood sugar utilization, even a moderate amount of sugar offered to them by the liver causes marked and prolonged hyperglycemia. The mere fact that blood sugar is of hepatic origin cannot explain the epinephrine hyperglycemia because it fails to take into account the quantitative relationship between blood sugar production and utilization.

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THE EFFECT OF EPINEPHRINE ON ARTERIAL AND VENOUS BLOOD SUGAR IN MEN.

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The experiments were performed on seven medical students of the University of Buffalo who volunteered. The subjects were instructed to come in the morning to the laboratory without breakfast. After a period of rest on a bed in a darkened room the first blood sample was removed, first from the radial artery and then without stasis from the cubital vein of the same side. While this was done the subjects were told to keep the arm as free from tension as possible. 1 cc. of the Parke, Davis 1:1000 adrenalin solution was then injected subcutaneously. Observations of reclining pulse rate and blood pressure were taken at frequent intervals and notes were kept of the subjective symptoms. 1 hour after the injection pulse rate and blood pressure had generally returned to normal and a second blood sample was removed from the radial artery and cubital vein. Each blood specimen was analyzed in triplicate by means of the Hagedorn and Jensen method (1). The titrations checked within 0.02 cc. of 0.005 N thiosulfate solution. The results obtained are recorded in Table I. With the exception of Subject Ki, the normal difference between arterial and venous blood sugar varied between 1 and 6 mg. This corresponds to the differences recorded in the literature and to data obtained previously in this laboratory on normal persons (2). In Subject Ki a certain nervous tension before the blood sampling was unmistakable, as shown by rapid changes in pulse rate. It is of interest that the reaction of this subject to adrenalin was unusually severe and that there was a marked tremor of the limbs at the time of the second blood sampling. Nevertheless this subject did not respond with the greatest increase in blood sugar after the injection. The other subjects

exhibited a quiet behavior throughout the experiment. A tremor of the limbs was noted in three other subjects (Subjects Ha, Ba, and Me). There was some individual variability in the increase in blood pressure and pulse rate and in the subjective symptoms therefrom. Subject Me, who is the only one of this group to respond with only a slight hyperglycemia, also showed a rise in pulse rate and blood pressure after the injection. Such increases in blood pressure as are observed in men (5 to 15 mg. of Hg) are not observed in the common laboratory animals after the subcutaneous injection of 30 times larger doses which points to a slower rate of

TABLE I.

Arterial and Venous Blood Sugar in Men after Subcutaneous Epinephrine Injections.

The results are expressed in mg. per 100 cc.

Subject.	Weight.	Before.			1 hr. after.		
		Radial artery.	Cubital vein.	Difference, A − V.	Radial artery.	Cubital vein.	Difference, A − V.
	lbs.						
Ha	168	97	95	2	164	162	2
Vo	175	113	107	6	171	168	3
McA	170	112	111	1	187	184	3
Ba	173	103	100	3	199	197	2
Ta	138	111	108	3	239	229	10
Ki	154	124	112	12	206	206	0
Me	156	108	104	4	120	119	1
Average		109.7	105.3	4.4	183.7	180.7	3

absorption of epinephrine from the subcutaneous tissue of rabbits, cats, and dogs than of men.

With the exception of Subject Ta, the increase in blood sugar after epinephrine injection is not accompanied by an increase in the arteriovenous blood sugar difference. If anything the differences are smaller than normal, since they vary between 0 and 3 mg. In only one case is the difference 10 mg. The significance of this result has been discussed in a previous paper (3) in which similar results were obtained on rabbits. The experimental results of Wiechmann (4) who determined plasma sugar in arterial and venous blood after epinephrine injections in men are in complete

accord with those here reported. Foster (5) was the first to make extensive comparisons of the blood sugar in capillary and venous blood and to use such differences as an index of blood sugar utilization in the peripheral tissues, notably muscles. He found, what has since been confirmed many times in men and animals, that the ingestion of glucose causes the arteriovenous blood sugar difference to rise to 30 mg. and more. Alimentary hyperglycemia is therefore followed by a more rapid uptake of blood sugar in muscle, while the excess of sugar accumulating in the blood after epinephrine injections does not evoke such a response.

SUMMARY.

Hyperglycemia following subcutaneous injections of 1 mg. of epinephrine in men is not accompanied by a rise in the arteriovenous blood sugar difference.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

V. ANALYSIS OF THE ACETONE-SOLUBLE FAT.*

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INTRODUCTION.

A number of publications dealing with the chemical composition of the lipoids of tubercle bacilli may be found in the literature but only fragmentary information regarding the composition of the true fats or glycerides is available. Practically all investigators who have worked on this subject agree in stating that the lipoids consist principally of phosphatides, wax, and glycerides but in no case reported in the older literature has an adequate separation into these components been accomplished. Lack of sufficient material as well as the lack of suitable methods evidently made such separation impossible. The fact must be borne in mind in considering the composition and constants of the lipoids that have been reported that all published data refer to analyses of mixtures containing varying proportions of phosphatides, wax, and glycerides or similar compounds.

The methods which were developed in this laboratory for the fractional extraction of tubercle bacilli and the separation of the lipoids (1) made it possible to prepare substances such as phosphatide, wax, and acetone-soluble fat or glycerides in relatively pure form. The fat used in our present investigation had been obtained as follows: Fresh living bacilli, Strain H-37, were extracted

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† Holder of the Milton Campbell Research Fellowship at Yale University, 1928–29.

with a mixture of alcohol and ether and the filtered extract was concentrated under reduced pressure until the greater portion of the solvents had been removed. The residual aqueous suspension of the lipoids was extracted with ether; the ethereal solution was concentrated and the phosphatide and wax were removed by treatment with acetone. The acetone-soluble fat, Fraction A-5, remaining in the mother liquor was obtained by evaporating the solution. The fat was saponified with alcoholic potassium hydroxide and the cleavage products were separated into the following groups: (*a*) unsaponifiable matter, (*b*) fatty acids, and (*c*) water-soluble constituents.

It is evident from our results that the solid unsaponifiable matter obtained from tubercle bacilli by other investigators had been derived from the wax fractions because substances such as mykol described by Tamura (2), the higher alcohols mentioned by Bürger (3), or the hyalinol of Goris (4) could not be found in the acetone-soluble fat. The small amount of unsaponifiable matter that we obtained was a liquid which did not solidify at 0° and gave no cholesterol or sterol color reactions. In this respect our observations are in agreement with the results of Kresling (5), Panzer (6), and Goris (4) who have reported that cholesterol could not be found in tubercle bacilli.

Hammerschlag (7), who published the first chemical investigation on tubercle bacilli, expressed the opinion that the fat consisted of a mixture of tripalmitin and tristearin with either none or very little triolein. DeSchweinitz and Dorset (8) believed that the fat contained lauric, palmitic, and arachidic acids, while Bulloch and Macleod (9) mention lauric, myristic, isocetic, and oleic acids, but Baudran (10) refers only to stearic and oleic acids. Goris (4) who had larger quantities of the lipoids at his disposal states that the fat consisted of free fatty acids, particularly oleic acid, and glycerides of butyric, caproic, palmitic, oleic, stearic, and arachidic acids together with liquid saturated fatty acids that could not be identified. The analysis of DeSchweinitz and Dorset (8) indicated that a small amount of volatile fatty acid was present and a similar observation was reported by Kresling (5) who regarded the volatile acid as butyric acid.

The mixed fatty acids that we obtained after saponification of the fat were separated by treating the lead soaps with ether

according to the Gusserow-Varrentrapp (11, 12) procedure into saturated solid acids and liquid acids. The saturated solid acids, amounting to 36 per cent of the total, consisted of palmitic acid, $C_{16}H_{32}O_2$, together with a smaller amount of stearic acid, $C_{18}H_{36}O_2$, and a very small quantity of cerotic acid, $C_{26}H_{52}O_2$. The various other fatty acids mentioned in the literature could not be found. The liquid acids, which constituted about 60 per cent of the total acids, had an iodine number of 53.8 which indicated that a large proportion of the liquid acids was saturated. The mixture of liquid acids was reduced with hydrogen and platinum oxide (13) and the reduction product was again separated by means of the lead soaps-ether treatment into solid saturated and liquid saturated acids. The reduced acid was found to be practically pure stearic acid, and we may conclude from the iodine number of the liquid acids and from the quantity of stearic acid obtained after reduction that the unsaturated acid is linoleic acid, $C_{18}H_{32}O_2$. The saturated liquid acids obtained from the ether-soluble lead soaps after reduction were separated by distillation in high vacuum into two principal fractions consisting of two new acids, tuberculostearic acid and phthioic acid, which will be more fully described in a subsequent paper.

The neutral bacillary fat is generally referred to in the literature as glycerides but the actual nature of the alcohol that is present has never been determined. The presence of glycerol in the mixed lipoids, as indicated by a positive acrolein test, has only been reported by Bulloch and Macleod (9), by Agulhon and Frouin (14), and by Koganei (15). During the present investigation a special effort was made to determine whether glycerol could be identified as one of the cleavage products after saponification of the fat. We obtained a thick, alcohol- and water-soluble syrup which gave a positive acrolein reaction but in an attempt to purify the substance by distillation under reduced pressure only a very small amount of a syrupy distillate went over, while the greater portion of the material decomposed and carbonized in the distillation flask at a comparatively low temperature. The distillate gave a positive acrolein reaction but when benzoylated by the method of Einhorn and Hollandt (16) we could not obtain the characteristic glyceryl benzoate. It is quite evident that the amount of glycerol present in the acetone-soluble fat is entirely

too small to account for the fatty acid esters as glycerides. It is very probable that the fatty acids are combined with some water-soluble alcoholic component other than glycerol, perhaps a carbohydrate that may have been changed or partly decomposed by the boiling alkali during the saponification.

It has been reported many times that a large proportion of free fatty acids is contained in the ether-soluble constituents. Our results fully corroborate these statements and we found that about 27 per cent of the fat was composed of free fatty acids. It is uncertain whether these acids occur as such in the bacteria or whether they are formed by decomposition or saponification as stated by Frouin and Guillaumie (17) but it would seem unlikely

TABLE I.

Constants of the Acetone-Soluble Fat.

	Found.	Reported by Kresling.	Reported by Goris.
Melting point	33°	46°	42°
Iodine No	52.6	9.82	16.20
Saponification No	203.6	60.7	124.4
Acid No	60.35	23.08	37.0
Ester No	143.25	37.62	87.4
Reichert-Meissl No	3.96	2.00	
Unsaponifiable matter	10.38		

that the mild methods which we employed in isolating the fat would cause such an extensive decomposition.

In view of the investigations of Sabin and Doan (18) upon the specific action of the lipoids and the liquid saturated fatty acids and the work of Mudd and Mudd (19) upon the surface structure of the bacilli which they believe to consist of a complex combination of protein and fats and possibly also carbohydrates, it seemed of interest to determine the nature of the free fatty acids. It was found that the free acids consisted of solid saturated, liquid saturated, and liquid unsaturated acids in nearly the same proportion as they are found in the total fat after saponification.

EXPERIMENTAL.

The acetone-soluble fat obtained as already described was a reddish brown soft mass with a peculiar but agreeable odor.

When slowly heated it melted at 33° after beginning to sinter at 25°, and when dried in a vacuum at 61° over dehydrite, it lost 1.42 per cent of its weight. The fat was free from nitrogen and sulfur and it contained only a very slight trace of phosphorus. The constants mentioned in Table I were determined according to the methods (20) of the Association of Official Agricultural Chemists, and for comparison, similar data published by Kresling and by Goris are included.

Saponification of the Fat.[1]

A total of 180 gm. of fat was saponified in portions of 25 gm. by refluxing for 5 hours with 300 cc. of 4 per cent alcoholic potassium hydroxide. The saponification mixtures were united, concentrated to about 1 liter, diluted with 3 liters of water, and extracted 7 times with ether. The ethereal extract, about 6 liters, was concentrated to dryness and the residue was again refluxed for 1 hour with alcoholic potassium hydroxide. The solution was diluted with water and extracted with three portions of ether and the ethereal solution was washed with water. The aqueous solution and the washings were added to the original soap solution.

The unsaponifiable matter was obtained by concentrating the ethereal solution and drying the residue. It formed a thick dark oil that weighed 21.5 gm.

The aqueous soap solution was acidified with hydrochloric acid and the fatty acids were extracted with three portions of ether; the ethereal solution, after being washed with water until free from hydrochloric acid, was dried over sodium sulfate, filtered, and the ether was distilled. The oily residue solidified to a soft crystalline mass on cooling to room temperature and after being dried in a vacuum desiccator it weighed 150 gm.

Examination of the Water-Soluble Constituents.

The dark colored aqueous solution, after the fatty acids had been extracted, was concentrated under reduced pressure to about

[1] All solvents used in this work had been freshly distilled and the alcohol had been purified by distillation over potassium hydroxide. During saponification and all subsequent operations nitrogen was used to displace the air as completely as possible from all vessels and solvents and during filtrations a stream of nitrogen was passed over the funnels.

200 cc., and the potassium chloride together with a small quantity of a dark brown tarry mass that had separated was removed by filtration. The filtrate was concentrated nearly to dryness in a vacuum and the moisture was removed as far as possible by evaporating the residue ten times with absolute alcohol. Glycerol and other alcohol-soluble substances were then removed by extraction with alcohol. The solution was filtered and again evaporated to dryness in a vacuum. After repeating these operations a very viscous dark brown alcohol-soluble syrup was obtained which had a peculiar odor. After drying in a vacuum desiccator the substance weighed 11.9 gm. A small quantity of the syrup was heated with acid potassium sulfate, when the acrid odor of acrolein was produced.

An attempt was made to purify the glycerol by distillation. At 190–200° and 30 mm. pressure a yellowish thick distillate went over which weighed 3 gm., but the balance of the material decomposed at the above temperature, forming a black carbonized mass in the distillation flask. The distillate was not homogeneous but contained some oily drops which were removed by extraction with ether. On evaporation of the ether about 1 gm. of a light yellow oil with an acrid odor was obtained which, for lack of time, we have not examined.

The ether-insoluble portion of the distillate, which presumably consisted of glycerol, gave a distinct acrolein reaction when heated with acid potassium sulfate, but we could not obtain any solid benzoyl derivative of the substance by the method of Einhorn and Hollandt. As judged by the acrolein reaction, the syrup undoubtedly contained some glycerol, but the amount is entirely too small to account for the fatty acid esters as glycerides.

The greater portion of the water-soluble constituents carbonized and decomposed at a temperature between 190-200° during the distillation mentioned above. It remains a problem for future research, therefore, to determine the nature of the principal alcoholic component of the tubercle bacilli fat.

Unsaponifiable Matter.

Lack of time has prevented a thorough examination of the unsaponifiable matter. As already mentioned, the substance formed a dark brown oil which did not solidify and nothing

separated from it either on cooling to 0° or on standing at room temperature. The Salkowski and the Liebermann-Burchard reactions were entirely negative, indicating the absence of any of the usual unsaturated sterols. It is noteworthy that the substance contains unsaturated compounds as indicated by the high iodine number of 85.9. We hope to complete the investigation of this material in the near future.

Separation of the Fatty Acids.

The remaining crude fatty acids, 144.3 gm., were converted into lead soaps, the latter were dried, extracted with ether, and the ether-soluble and the ether-insoluble portions were decomposed by shaking with dilute hydrochloric acid. The solid fatty acids obtained in this manner weighed 52.6 gm. and the iodine number was 3.5. The small amount of adhering unsaturated acids was lost on crystallization from alcohol. The liquid fatty acids formed a thick dark brown oil that weighed 87.7 gm. and the iodine number was 53.8.

Examination of the Solid Fatty Acids.

It was found in a preliminary experiment that it was impossible to effect a complete separation of the solid fatty acids by crystallization from methyl or ethyl alcohol, acetone, or benzene. After three crystallizations, the melting point remained constant at 56–58° and the molecular weight was about 270.

Isolation of Cerotic Acid.

The total crude solid acids were dissolved in 500 cc. of hot alcohol, treated with norit, filtered, and the norit was washed with 200 cc. of hot alcohol. The solution on cooling to room temperature deposited 1.5 gm. of crystals, m.p. 78–80°, and after standing overnight an additional 1.5 gm. of substance, m.p. 66–73°, had separated. These fractions were recrystallized several times from benzene and from acetone and the melting point rose gradually to 84–85°. The substance which now weighed 1.5 gm. was recrystallized twenty times from benzene and from acetone but there was no change in melting point. On analysis the following values were found.

0.1127 gm. substance: 0.1332 gm. H_2O and 0.3273 gm. CO_2.

Found.	C 79.21, H 13.23.
Calculated for $C_{26}H_{52}O_2$(396).	" 78.78, " 13.13.

Titration.—0.2826 gm. and 0.3432 gm. of substance were dissolved in neutral alcohol and titrated with 0.1 N alcoholic potassium hydroxide, with phenolphthalein as indicator. Required, 7.16 cc. and 8.70 cc. of 0.1 N KOH.

Found.	Mol. wt. 394.7, 394.5.
Calculated for $C_{26}H_{52}O_2$.	" " 396.

The silver salt was prepared by adding silver nitrate to the alcoholic solution of the potassium salt. The precipitate was filtered, washed, and dried.

0.2754 gm. substance: 0.0605 gm. Ag.

Found.	Ag 21.97.
Calculated for $C_{26}H_{51}O_2Ag$(502.88).	" 21.45.

The analytical values found indicate that this high melting acid was cerotic acid, $C_{26}H_{52}O_2$.

Isolation of Palmitic and Stearic Acids.

As has been mentioned above, it was impossible to separate the major portion of the fatty acids by crystallization and it was found to be equally impossible to effect any separation by means of fractional precipitation with magnesium acetate. The acid was separated into ten fractions in this manner but the melting points of the different fractions only varied between 53–58°.

The mixed acids which remained after removing the cerotic acid were therefore converted into the methyl esters and the latter were subjected to fractional distillation in a vacuum of 0.01 to 0.001 mm. pressure. The distillates were colorless and crystallized at room temperature. The following fractions were collected.

Fraction No.	Temperature of air bath.	Boiling point of ester.	Weight.
	°C.	°C.	gm.
1	140–150	110–120	1.8
2	150–155	120–130	24.8
3	155–170	125–135	10.7
4	170–180	140–142	3.6
5	Residue in distillation flask.		2.6

Fractions 1 and 2 were united and again distilled under the same conditions as noted above. The temperature of the air bath and the boiling point of the ester were alike and varied between 115–116°. The distillate which went over at this temperature, Fraction 1a, weighed 12.2 gm. and melted at 29°.

Fraction 3 was then added to the residue in the flask and the distillation was continued but in a somewhat lower vacuum. Two intermediate fractions, Fractions 1b and 1c, amounting to 24.1 gm. were collected between 127–132° and 132–137° and finally Fraction 1d went over between 163–165°. It weighed 1.9 gm. and melted at 34°.

Fraction 1a was saponified with alcoholic potassium hydroxide and the free acid was isolated and twice recrystallized from methyl alcohol. Snow-white plates were obtained that melted at 63–64° and after two more recrystallizations from acetone the melting point had not changed. When mixed with pure palmitic acid, there was no depression of the melting point.

0 2622 gm substance: 0 2901 gm. H_2O and 0.7173 gm. CO_2.

Found.	C 74.61,	H 12.38.
Calculated for $C_{16}H_{32}O_2$(256).	" 75.00,	" 12.50.

Titration.—0.4990 gm. of substance was dissolved in neutral alcohol and titrated with 0.1 N sodium hydroxide, with phenolphthalein as indicator. Required, 19.63 cc. of 0.1 N NaOH.

Found.	Mol	wt.	254.2.
Calculated for $C_{16}H_{32}O_2$	"	"	256.

Analysis of the ester, Fraction 1a, was as follows:

0 2135 gm. substance: 0.2440 gm. H_2O and 0.5909 gm. CO_2.

Found.	C 75 48,	H 12.79.
Calculated for $C_{16}H_{31}O_2CH_3$(270).	" 75.55,	" 12.59.

It is evident from the above data that the first fraction of the ester that distilled over consisted of practically pure methyl palmitate from which pure palmitic acid was obtained on saponification.

Fraction 1d and Fraction 4 were saponified in the manner just described and the free acid was isolated, yielding snow-white crystals from alcohol and acetone. The substances melted at

69–70° and at 67–68° and when mixed with pure stearic acid, m.p. 69-70°, there was no depression.

0.1936 gm. substance:	0.2223 gm. H_2O and 0.5415 gm. CO_2.		
0.1546 " "	: 0.1793 " " " 0.4321 " "		
Found.		C 76.28, 76.22,	H 12.85, 12.98.
Calculated for $C_{18}H_{36}O_2$(284).		" 76.05,	" 12.67.

Titration.—The acid was dissolved in neutral alcohol and titrated with 0.1 N sodium hydroxide, with phenolphthalein as indicator.

0.2974 gm. substance:	10.38 cc. 0.1 N NaOH.	
0.2520 " "	: 8.78 " 0.1 " "	
Found.		Mol. wt. 286.5, 287.
Calculated for $C_{18}H_{36}O_2$.		" " 284.

The above data indicate that the highest boiling fractions of the ester consisted of practically pure stearic acid.

The residue from the first distillation, Fraction 5, was saponified and the free acid was isolated. After two crystallizations from methyl alcohol and one from acetone, the crystals melted at 75°. Recrystallizing from benzene and from alcohol raised the melting point 76–77°, and after ten recrystallizations from benzene and one from acetone the melting point was 82–83°. It is probable therefore that this last fraction of the ester contained a small amount of cerotic acid.

Since the first fraction of the distilled esters contained palmitic acid and the last fraction with the highest boiling point contained stearic acid, we believe that the intermediate fractions must have consisted of the same acids. In other words we found no evidence of the presence of lower saturated fatty acids such as caproic, lauric, or myristic acid and moreover it is very probable that the arachidic acid reported by other investigators represented impure specimens of cerotic acid.

Examination of the Liquid Fatty Acids. Reduction of the Unsaturated Acid.

The liquid fatty acids, as already mentioned, formed a dark brown oil. The mixture contained some substance which acted like an indicator. When the acids were dissolved in alcohol and

made slightly alkaline, the solution turned a deep purplish red color. This substance, as well as the dark colored impurities, was removed with great difficulty. About fifteen treatments of an ethereal solution of the acids with norit in the presence of hydrochloric acid were required to remove the coloring matter. The crude acids could not be reduced with hydrogen and platinum oxide because the impurities poisoned the catalyst. Even after the acids had been decolorized with norit reduction was very slow and it was necessary to add fresh portions of catalyst several times before the iodine number was 0.

After complete reduction had been attained, the reduced acid was separated by means of its ether-insoluble lead soap. The lead soap-ether treatment was repeated five or six times before the reduction product and the substance which acted as an indicator had been removed completely from the liquid saturated acids.

The reduced acid was isolated in the usual manner by shaking the lead soap with dilute hydrochloric acid and ether, washing with water, evaporating the ether, and drying the residue. The white solid substance weighed 22.1 gm., corresponding to 25.4 per cent of the total liquid acids. The acid was recrystallized three times from methyl alcohol and twice from acetone. The snow-white crystals melted at 68 69°, solidified at 66°, and there was no depression when mixed with pure stearic acid.

0 1853 gm substance: 0 2128 gm. H_2O and 0 5183 gm. CO_2.

Found	C 76 28,	H 12 85.
Calculated for $C_{18}H_{36}O_2$(284)	" 76 05,	" 12 67.

Titration.—0.4474 gm. of substance dissolved in neutral alcohol with phenolphthalein as indicator required 15.71 cc. of 0.1 N NaOH.

Found	Mol wt. 284 8.
Calculated for $C_{18}H_{36}O_2$.	" " 284.

The values reported above indicate that the reduction product was stearic acid. It must be mentioned, however, that a small quantity of acid was isolated from the mother liquor which melted at 56 58° and solidified at 54–55°. The reduction product contained, therefore, some acid which lowered the melting point

of stearic acid. This effect might be due, however, to a slight contamination with the liquid saturated acids.

When we consider the amount of reduced acid obtained in comparison with the iodine number of the liquid acids, the conclusion seems justified that the unsaturated acid consisted principally of linoleic acid, $C_{18}H_{32}O_2$. The total liquid acids that were reduced weighed 86.7 gm. and the iodine number of 53.8 would require about 59 per cent of oleic acid, 29 per cent of linoleic acid, and 19 per cent of linolenic acid. The reduced acid, 22.1 gm., corresponding to 25.4 per cent of the total acids, agrees most closely with the calculated quantity of linoleic acid.

As a further confirmation we prepared a bromine derivative of the unsaturated acid. 1 gm. of the liquid acid was dissolved in 15 cc. of petroleum ether and while cooling in a freezing mixture a solution of 1 gm. of bromine in 10 cc. of petroleum ether was added slowly. A precipitate separated immediately and after standing for some time in the freezing mixture it was filtered, washed with cold petroleum ether, and dried. The dark brown amorphous powder weighed 0.26 gm. The substance was easily soluble in benzene but not entirely soluble in ether and it was insoluble in petroleum ether. It could not be obtained in crystalline form. When heated in a capillary tube, the crude precipitate melted not sharply at 112–113°. The melting point of the bromine derivative of linoleic acid, $C_{18}H_{32}O_2Br_4$, is 114°.

Examination of the Liquid Saturated Acids.

The ether-soluble lead soaps obtained after reducing the unsaturated acid were decomposed by shaking with dilute hydrochloric acid, and the lead chloride and excess of hydrochloric acid were removed by washing with water. The ethereal solution was dried with sodium sulfate, treated several times with norit, filtered, and the ether was distilled. The residue after drying in a vacuum desiccator formed a brown oil that weighed 49.1 gm. The acids were converted into methyl esters and the latter were fractionally distilled in high vacuum when two principal fractions were obtained. These fractions consisted of two new fatty acids, tuberculostearic acid and phthioic acid, which will be more fully described in a subsequent publication.

Examination of the Fat for Free Fatty Acids.

The fat, 5.1 gm., was dissolved in 400 cc. of ether and the solution was shaken with two portions of 200 cc. of 1 N sodium hydroxide. The dark colored alkaline solution was twice extracted with ether, acidified with hydrochloric acid, and again extracted with ether; the ethereal solution was washed with water, filtered, and the ether was distilled. The residue was dissolved in alcohol, neutralized with potassium hydroxide, diluted with water, extracted with ether, and the aqueous solution was precipitated by adding an excess of lead acetate. The lead soaps were filtered, washed with water, dried, and extracted with ether.

TABLE II.

Constituents of the Acetone-Soluble Fat.

	Per cent of fat.	Per cent of fatty acids.
Water-soluble compounds	6.61	
Fatty acids	83.33	
Unsaponifiable matter	11 94*	
Free fatty acids calculated as palmitic acid	27.53	
Neutral esters	60.59	
Solid fatty acids	30 38	36.45
Liquid " "	50.65	60.78
Unsaturated fatty acids	12.59	15.11
Saturated liquid fatty acids	38 06	45.67

The Reichert-Meissl number would indicate some volatile acid but we were unable to isolate such an acid after saponification. But it is not improbable that a trace of butyric acid is present since from another fraction of mixed lipoids we have actually isolated a small quantity of a volatile acid with an odor similar to that of butyric acid.

* The substance had been dried in a vacuum desiccator, hence the value is slightly higher than that found on drying at 100°.

The ether-insoluble lead soaps yielded 1.1 gm. of solid saturated acids which after three recrystallizations from methyl alcohol melted at 56–57°. On titration 0.2291 gm. of substance required 8.55 cc. of 0.1 N NaOH. Found, mol. wt. 268.

The melting point and neutralization value indicate a mixture of palmitic and stearic acids very similar to the mixed solid acids that were isolated from the fat after saponification.

The liquid fatty acids were isolated from the ether-soluble lead salts and formed a brown oil that weighed 0.8 gm. The iodine number, which was 47.4, indicates that the proportion of unsaturated and liquid saturated fatty acids was nearly the same as that found in the total fat.

In conclusion there is presented in Table II a summary of the results of our analysis of the acetone-soluble fat.

SUMMARY.

1. The acetone-soluble fat derived from the human type of tubercle bacilli, Strain H-37, consists of free fatty acids and fatty acid esters of higher alcohols but only a small amount is present as glycerides. The nature of the principal alcoholic component could not be determined, but it is probably a polyhydric alcohol belonging to the carbohydrates.

2. The unsaponifiable matter does not contain cholesterol or any substance giving sterol color reactions.

3. The fatty acids consist of solid saturated acids, unsaturated acids, and liquid saturated acids.

4. The solid saturated fatty acids consist principally of palmitic acid, together with a small quantity of stearic acid and a very small amount of cerotic acid.

5. The unsaturated acid is most probably linoleic acid.

6. The liquid saturated fatty acids constitute about 45 per cent of the total acids and they represent the important biologically active constituents of the fat.

7. The free fatty acids are present in practically the same proportions as they occur in the total fat.

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FURTHER OBSERVATIONS ON THE OCCURRENCE OF PROTOCATECHUIC ACID IN PIGMENTED ONION SCALES AND ITS RELATION TO DISEASE RESISTANCE IN THE ONION.*

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INTRODUCTION.

In a paper recently published in this *Journal* (1) we reported the isolation of the phenolic acid commonly known as protocatechuic acid (3,4-dihydroxybenzoic acid) from the outer bulb scales of pigmented onions. The occurrence of this acid in the outer scales has a significant relationship to disease resistance in the onion. Protocatechuic acid is apparently one of the chemical entities that enables pigmented onions to resist the invasion of *Colletotrichum circinans* (Berk.) Vogl., the fungus organism responsible for the disease commonly known as *smudge*.

In view of the fact that the white varieties of onion are susceptible to the disease and as far as our chemical investigations have been able to show do not contain protocatechuic acid in the outer bulb scales, it appears that we have established for the first time a specific chemical difference between a resistant host (the pigmented onion) and a non-resistant host (the white onion). We emphasized that the toxicity experiments conducted along with the chemical investigations indicated that not all of the inherent

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toxicity exhibited by a definite unit of the aqueous extracts from the pigmented scales could be attributed to protocatechuic acid on the basis of the quantity of the acid that has been isolated by the method employed. It is reasonable to suppose that some of the acid may have been lost in the process of isolation and purification. The quantitative recovery of protocatechuic acid from the complex substances that are extracted with it is obviously beset with difficulties. On the other hand, we have not overlooked the possible presence of other compounds closely related to protocatechuic acid that may contribute to the resistance of the pigmented onion.

In this paper we report the progress made toward the objective of obtaining a better method of extracting and isolating the protocatechuic acid. Through the use of improved experimental methods that differ decidedly from the original we have been able not only to obtain larger yields of the acid, but we have also been able to obtain the acid from bleached pigmented scales from which originally it could not be isolated. This is also an opportune time to indicate that through the employment of the improved methods, we have been able to correlate certain field observations of the symptoms and seasonal development of the disease with the chemical data.

Correlation of Smudge Disease with Pigment Occurrence and Presence of Protocatechuic Acid.

Field studies conducted by one of us (2) showed that the resistance exhibited by the pigmented onion to the smudge disease is not an absolute resistance and that it is directly associated with pigment occurrence and pigment content. The pigments in colored varieties of onions appear in the outer bulb scales or leaves when the plants are about half grown or a little earlier. With age the coloring becomes more intense until a short time before maturity when the red or yellow pigments, depending on the variety, are fully developed. At this period the plants have one to several thin papery outer scales which are intensely colored. At the end of the growing season there is a tendency among the colored varieties toward some pigment reduction in the outer scales, especially in the portions above the soil line. The red and yellow pigments of onion scales are solutes in the cell sap of

the outer epidermal layers. Upon death of the cells in the dry outer scales, the cell sap pigments can diffuse out readily. This diffusion is particularly marked when the onion bulbs are unduly exposed after maturity to the bleaching action of direct sunlight, and to the leaching effect of meteoric and soil water. Ordinarily, susceptibility to the disease in the colored onion is limited to those regions near the top of the neck of the bulb where there is little or no pigmentation and in rare occasions to those colored bulbs that are unduly exposed to the leaching action of meteoric and soil water.

One of the purposes of this study was to determine whether the bleaching of the pigment and increase in susceptibility to infection were correlated with a simultaneous loss of protocatechuic acid from the outer scales. The protocatechuic acid content of the pigmented scales varies considerably. It is apparently absent or at least present only in minute amounts in the unpigmented portions of the colored onion bulbs (that is at the top of the bulb and near the neck) and is present in the greatest quantity in those outer bulb scales that show the highest degree of pigmentation. Furthermore it varies with the status of pigment preservation. Scales that have been badly bleached have a much lower protocatechuic acid content than fresh well preserved scales. Protocatechuic acid is much more soluble than the pigments with which it is associated in the colored scales. The relatively high solubility of protocatechuic acid in water (1 to 55 parts at 14°) indicates that the slow but certain leaching action of meteoric and soil water would tend to reduce the concentration of the acid. In fact some lots of red scales which had been subjected to considerable leaching yielded only minute traces of the acid, although there was still considerable pigment present. These facts are therefore in accord with the observations noted above and indicate that infection of uncolored portions of pigmented bulbs near the neck and of unduly exposed colored scales is due in part at least to the removal of protocatechuic acid through leaching.

Although it is a safe generalization to state on the basis of field observations that the red onion is as resistant to the smudge disease as the yellow onion, we have so far found the highest concentration of protocatechuic acid in yellow scales. The general average yield of the acid from bleached scales has, however, been

higher from red bleached scales. From repeated experimental trials we have learned that the isolation of the protocatechuic acid in the pure state, m.p. 199°, can be accomplished more readily from the extracts of the yellow scales. Protocatechuic acid can be readily separated from quercetin, the pigment of the yellow onion, because of the relatively high insolubility of quercetin in water. However, a portion of the pigments in the red scales is appreciably more soluble in water than is quercetin. The last traces of the red pigment can be removed only with difficulty from a solution that contains protocatechuic acid. The necessity of having to decolorize the extracts of the red onion scales through the repeated use of adsorbing agents probably tends to reduce the quantity of protocatechuic acid that can be obtained in the crystalline condition.

Improved Method of Isolating Protocatechuic Acid.

It soon became apparent that several aspects of the method originally employed in the isolation of the acid were contributing to irregular and low yields. By extracting the scales with water at 30°, as was done in the original method, a gummy substance of a pectin-like nature is removed, which is in part precipitated with lead acetate and passes into solution again when the lead precipitate is decomposed. This substance interferes with the crystallization of the acid. It was also noticed that when the voluminous reddish brown lead precipitate obtained by direct precipitation of the aqueous extract was decomposed with hydrogen sulfide, a considerable portion of the coloring matter was removed by the heavy lead sulfide precipitate. It therefore seemed quite probable that the freshly formed lead sulfide was not only removing coloring matter but also some of the protocatechuic acid by adsorption, which subsequently could not be removed by washing with water. The efficacy of freshly precipitated lead sulfide as an adsorbent of organic coloring principles has been observed before. Stenhouse (3) employed it to remove the coloring matter formed in the fusion of East Indian kino with alkali, from which he first isolated protocatechuic acid. The procedure herein reported eliminates the two sources of loss mentioned above. The new method has not only increased the yield of the acid but has also given more constant yields from the same scales. It has also enabled the isolation

of minute quantities of protocatechuic acid from scales that had been strongly leached by the action of meteoric and soil water.

The pigmented onion scales dried at 80° are extracted for 24 hours with dry acetone in a large type of Soxhlet extractor devised by Sando (4). The acetone extract is concentrated to dryness in a vacuum of 15 mm. pressure at 30°. The residue is taken up with water, whereby an almost complete separation of the water-soluble substances from the quercetin and a dark gum-like substance is effected. To the aqueous solution, which contains practically all of the protocatechuic acid and some pigment impurities other than quercetin, basic lead acetate is added, an

TABLE I.

Yields of Pure Protocatechuic Acid in Gm. per 100 Gm. of Scales.

Status of pigment or pigment preservation.	Variety of onion scales.	
	Red.	Yellow.
Fresh unbleached scales (maximum pigmentation)	0.08–0.125*	0.08–0.135*
Unpigmented portions from top and neck of bulb	0.01–0.03	0.00–0.02
Partially bleached	0.07–0.10	0.05–0.08
Badly bleached	0.02–0.06	0.00–0.03

* The toxicity control experiments indicate that the maximum yield of protocatechuic acid so far obtained accounts approximately for 35 to 40 per cent of the toxicity of the extracts from which the acid was isolated.

excess being avoided, until complete precipitation is accomplished. The reddish brown lead precipitate is filtered off, washed with cold water, and decomposed with sulfuric acid. The decomposition is carried out by suspending the lead precipitate in a large volume of water at 85–90° while dilute sulfuric acid (about 10 per cent) is slowly added from a burette. The solution is kept in constant agitation by means of a mechanical stirrer. Only a minute excess of sulfuric acid is added. The end-point is determined by taking out small test portions to establish the requisite quantity of acid needed to decompose the precipitate in the entire solution. After the lead sulfate has settled out, the supernatant liquor is filtered off and concentrated to a small volume under diminished pressure at 45°. The resulting syrup which is usually

still highly pigmented is then taken up in acetone, separated from any insoluble matter that remains, and boiled with activated blood charcoal under a reflux condenser. After the charcoal is filtered off, the solution is again concentrated to about one-third of its volume under reduced pressure. The solution is then transferred to an open vessel and allowed to stand uncovered at room temperature, whereupon in the course of 48 hours the protocatechuic acid usually crystallizes out in a crude form. To remove the last traces of coloring matter the crude crystals are dissolved in hot water and shaken with a suspension of freshly precipitated lead sulfide. After removing the lead sulfide and again concentrating to a small volume, the protocatechuic acid crystallizes out in the usual form as pale yellow monoclinic needles, m.p. 199°.

In Table I the data obtained through the use of the new method are given. The concentrations of protocatechuic acid in fresh unbleached scales, the unpigmented portion from the top and neck of the bulb, and from bleached scales are given for comparison. The lowest and highest yields obtained are given in each case to show the range of the occurrence of the acid. The values presented represent the minimum and maximum mean, respectively, secured from four separate extractions.

SUMMARY.

1. A more refined and accurate procedure for the isolation of protocatechuic acid from pigmented onion scales is given.

2. The quantity of protocatechuic acid in mature pigmented onion scales varies considerably and correlates closely with the status of preservation of the pigments in the scales, being highest in those scales in which the pigment preservation is at a maximum.

3. The unpigmented portions of the colored onion scales (those regions near the top and neck of the bulb) either have only a small quantity of the acid or the acid is not present.

4. Pigmented scales that have been badly leached by the action of meteoric or soil water, although some pigment may still be intact, contain little or no protocatechuic acid.

5. All of the results so far obtained on the occurrence of protocatechuic acid in the outer scales of the pigmented onion (the

so called resistant types), correlate with the field observations made with reference to the occurrence and development of the smudge disease.

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THE EFFECT OF VITAMIN DEFICIENCIES ON CARBOHYDRATE METABOLISM.

II. THE INFLUENCE OF UNCOMPLICATED VITAMIN B DEFICIENCY ON CONCENTRATION OF TRUE SUGAR, REDUCING NON-SUGAR, AND ALKALINE RESERVE IN THE BLOOD OF THE ALBINO RAT.*

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In a recent communication (1) we reported hypoglycemia in nursing young of the albino rat suffering from uncomplicated vitamin B deficiency. In this investigation we have extended our observations to weaned and adult rats. After the work was already in progress Somogyi's method (2) of removing non-fermentable reducing substances together with the proteins appeared, and we then began to accumulate data on true as well as apparent sugar. We used 0.1 cc. of peripheral blood, and found Folin's modified micro ferricyanide method (3) most suitable for our purpose. In order to apply Somogyi's method of removing proteins and reducing non-sugars to the Folin micro blood sugar method, we found the following concentrations of reagents most satisfactory: 0.1 cc. of blood, 1.6 cc. of the zinc sulfate ($ZnSO_4 \cdot 7H_2O$) reagent, 0.2 cc. of 0.15 N NaOH, 0.1 cc. of distilled water. Pure glucose in concentrations found in our work was quantitatively recovered in many trials in the zinc sulfate filtrates by employing the concentrations of reagents outlined above.

The literature on the effect of vitamin B deficiency on the concentration of blood sugar has been recently reviewed by Stucky and Rose (4) who disclose conflicting evidence. The most recent contribution, involving the rat as the experimental animal, is

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that of Drummond and Marrian (5) who state that "there appears to be a definite hyperglycæmia in the rat during the greater part of the period it is kept on a ration deficient in vitamin B" and that "there is a very marked fall in the blood sugar level during the final stage of decline when the body temperature and metabolism are reduced." We show in this paper that the hyperglycemia frequently encountered in vitamin B deficiency is in the apparent, but not in the true sugar; *i.e.*, the disturbance is in the reducing non-sugars. In addition to blood sugar, we have also studied the influence of polyneuritis on alkaline reserve of the blood. We employed Van Slyke and Neill's manometric method on samples of 0.1 cc. of plasma (6). Our previous findings of anhydremia (7) in vitamin B deficiency stimulated a detailed study of the water intake, and a summary of such results is also submitted.

A total of thirty-six animals was employed, half of which served as controls. Of these, twenty-four were adult females, and twelve mixed sexes which were taken at weaning. Our findings, which are given only for those animals on which we have complete records (fourteen control and fifteen pathological) are summarized in Tables I to VII. In Charts I to III are given illustrations (one normal and two pathological) showing the concentration of apparent and true blood sugar as well as the daily records of food consumption and water intake. The adult animals were transferred from our Stock Diet 5, which is a modification of Sherman and Muhlfeld's ration (8), composed of whole wheat, 60.7; rice polish, 5.0; whole milk powder, 33.0; and NaCl, 1.3. These animals received daily 6 drops of cod liver oil as a supplement to Rations 1202 and 1452, the composition of which is given later in the paper. The weaned animals were transferred from one of our stock diets containing cod liver oil, and, therefore, had enough storage of vitamins A and D, so that the 5 per cent of butter fat in their ration supplied all the necessary fat-soluble vitamins for growth.

Shortly after this study was initiated we began to anticipate marked rises in the blood sugar level which follow a meal (9), and, therefore, made bleedings 2 hours after fasting. In order to secure reliable information on the influence of the extent of fasting on the concentration of true and apparent blood sugar, we carried out a number of experiments, the results of which are submitted

TABLE I.

Effect of Fasting on Concentration of Blood Sugar of Albino Rats.

Animal No.	Age.	Weight before fast.	Weight after 24 hr. fast.	Concentration of apparent sugar, mg. per cent.						Concentration of true sugar, mg. per cent.					
				0 hr.	1 hr.	3 hrs.	5 hrs	7 hrs.	24 hrs.	0 hr.	1 hr.	3 hrs.	5 hrs	7 hrs.	24 hrs.
	days	*gm*	*gm.*												
♀ 6008	68	137	127	128	125	121	101	72	70	91	92	98	80	53	51
♀ 6009	72	146	136	128	130	109	99	98	94	94	94	80	78	74	72
♂ 6010	87	181	171	104	124	110	130	100	92	78	96	85	102	74	67
♂ 6011	84	223	208	124	116	133	103		93	91	85	97	78		61
♂ 6012	89	271	256	124	122	116	133		98	93	91	87	108		72
♀ 6013	89	143	134	131	129	118	129		95	96	94	88	95		69
♀ 5718	290	176	165	123	121	110	98	98	92	97	92	84	73	70	68
♀ 5718	296	171	162	113	116	115	95	83	86	87	87	86	73	65	62
♀ 5813	371	163	154	124	126	103	124	99	93	94	94	75	96	73	71
♀ 5732	445	216	204	122	114	116	133	75	85	91	85	92	100	58	64
♀ 5757	281	188	178	108	109	109	97	95	98	84	84	80	75	66	71
♀ 5757	296	202	189	121	144	103	94	66	72	90	110	80	73	50	52

TABLE II.

Concentration of Blood Sugar in Growing and Adult Albino Rats (Control Groups on Ration 1202).

Animal No.	Age.	Weight.	Apparent sugar.		True sugar.		Reducing non-sugar substances.	
			Maximum.	Minimum.	Maximum.	Minimum.	Maximum.	Minimum.
	days	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
♀ 6008	36– 89	60–170	131	117	106	94	31	24
♀ 6009	36– 91	68–146	133	115	105	86	35	23
♂ 6010	36– 90	72–186	141	108	104	82	31	22
♂ 6011	36– 91	73–223	132	105	109	79	34	21
♂ 6012	36– 90	82–271	148	116	115	88	33	22
♀ 6013	36– 90	57–143	139	108	110	84	31	24
♀ 5718	234–278	167–175	132	100	100	73	38	22
♀ 5812	311–320	195–196	130	95	100	72	33	30
♀ 5814	178–192	169–171	143	100	103	75	40	25
♀ 5751	313–327	236–220	130	100	96	74	34	26
♀ 5732	420–438	217–218	118	100	85	73	33	27
♀ 5756	197–235	241–252	135	111	100	83	35	22
♀ 5757	229–278	190–193	145	105	116	84	37	21
♀ 5765	255–294	228–208	115	100	87	73	28	27

in Table I. It will be noted that the lowest figures in both the apparent and true sugar are encountered at the end of a 7 hour fast, and that the fasting hypoglycemia is not accentuated after 17 hours additional fasting. An examination of Table II indicates that the concentration of reducing non-sugars in the albino rat, 36 to 438 days of age, during growth or maintenance is 21 to 40 mg. per cent. Table III shows the rise in the reducing non-sugars in

TABLE III.

Effect of Uncomplicated Vitamin B Deficiency on Concentration of Blood Sugar.

Animal No.	Age.	Weight.	Apparent sugar.		True sugar.		Reducing non-sugar substances	
			Maximum.	Minimum.	Maximum.	Minimum.	Maximum.	Minimum.
	days	*gm.*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
♂ 6002	35– 85	67– 60	175	95	104	72	90	15
♂ 6003	35– 63	53– 43	131	80	104	62	46	15
♀ 6004	35– 93	51– 62	152	96	108	73	44	19
♀ 6005	35– 93	63– 51	143	100	101	70	45	18
♂ 6006	35– 86	62– 47	139	60	112	43	43	20
♀ 6007	35– 91	55– 61	133	93	95	74	49	12
♀ 5701	321–330	165–142	225	122	158	89	67	33
♀ 5738	418–422	136–124	133	108	98	81	37	27
♀ 5737	400–417	176–155	133	110	100	77	33	28
♀ 5736	252–279	126– 96	133	93	104	62	36	26
♀ 5748	329–346	158–149	133	113	95	74	43	19
♂ 5801	91–103	68– 62	156	75	78	75	81	30
♀ 5805	82– 91	107– 96	138	108	102	81	36	27
♀ 5806	82– 91	53– 41	124	93	94	72	30	21
♀ 5817	72– 77	60– 40	148	119	111	76	49	23

uncomplicated vitamin B deficiency, ten out of fifteen animals showing such increase. Three animals gave a concentration of reducing substances other than true glucose of 67, 81, and 90 mg. per cent respectively. On the other hand, we found no disturbance in the concentration of true sugar until the latter stages of avitaminosis, associated with inanition, when a hypoglycemia is apparent to the extent observed after a 7 hour fast.

Chart I shows the range of concentration of apparent and true

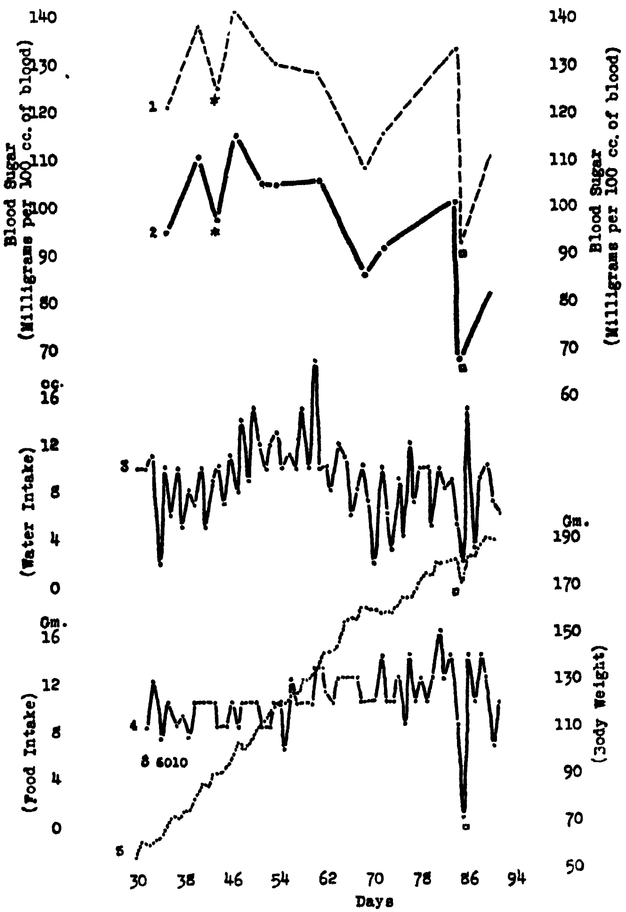

CHART I. The concentration of apparent and true blood sugar in a control, growing animal, 30 to 91 days of age. From point * on the peripheral bleedings were made 2 hours after fasting. At point □ the animal was fasted for 24 hours. Curve 1 represents apparent blood sugar in mg. per cent; Curve 2 represents true blood sugar in mg. per cent; Curve 3 represents daily water intake in cc.; Curve 4 represents daily food consumption in gm.; Curve 5 represents body weight in gm.

blood sugar in a control animal making excellent growth on our Ration 1202.[1] It also shows the effect of a 24 hour fast on the same animal.

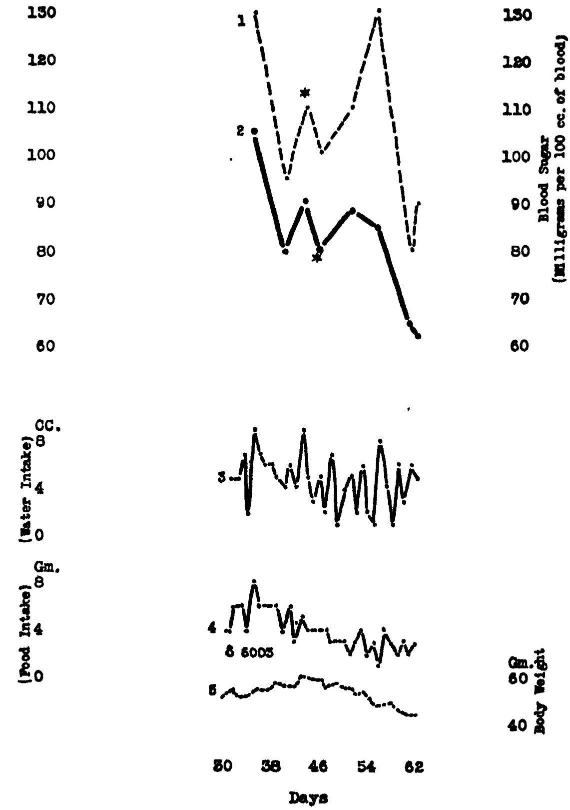

CHART II. The concentration of apparent and true blood sugar in an animal suffering from uncomplicated vitamin B deficiency, 30 to 62 days of age, showing loss of body weight and incipient posterior paralysis during the last 2 weeks of the experiment. From point * on the peripheral bleedings were made 2 hours after fasting. Curve 1 represents apparent blood sugar in mg. per cent; Curve 2 represents true blood sugar in mg. per cent; Curve 3 represents daily water intake in cc.; Curve 4 represents daily food consumption in gm.; Curve 5 represents body weight in gm.

[1] Composition of Ration 1202: casein (purified) 20, Salt Mixture 185 (10) 4, Northwestern yeast 5, butter fat 5, dextrin 66.

Chart II gives a typical illustration of the blood sugar curves encountered in vitamin B deficiency on Ration 1452.[2] While the apparent sugar concentration is rising appreciably, the true sugar is falling. The final hypoglycemia is undoubtedly due to marked inanition. It will be noted that the water intake is frequently reduced to as little as 1 cc. daily. The tremendous fluctuations in the daily water intake, while also found among the control animals (Chart I) takes place in the polyneuritic cases at a lower level.

Chart III shows the most marked case out of three in which the concentration of reducing non-sugars rose above 60 mg. per cent. This case was selected to illustrate the possibility of the concentration of apparent sugar increasing tremendously without any accompanying change in the concentration of true sugar. The non-reducing sugars reached a level of 90 mg. per cent. These non-fermentable reducing substances in the blood, according to Somogyi (11) may be due largely to glutathione and ergothioneine. Our conclusions, then, are that the hyperglycemia frequently reported in the literature in a deficiency of the vitamin B complex most probably is due to increases in reducing non-sugars and not to fermentable glucose or true sugar. Our results on adults and weaned animals are at variance with those found in nursing young of the albino rat (1). In the latter seventeen out of forty-four cases suffering from uncomplicated vitamin B deficiency showed a progressive hypoglycemia (in apparent sugar) during a period of prolonged maintenance before the onset of accentuated polyneuritic symptoms. Since, however, nursing young have a considerably greater calorie requirement in proportion to body weight than weaned or adult animals, it is quite possible that the insufficient food intake on the part of the lactating mother resulted in insufficient carbohydrate available to the nursing young for the production of a normal blood sugar concentration. At any rate, in weaned and adult albino rats we do not encounter any hypoglycemia until marked inanition takes place.

We also find that in uncomplicated vitamin B deficiency there occurs a pronounced reduction in the glycogen content of the liver,

[2] Composition of Ration 1452: same as Ration 1202 with the exception that the yeast was autoclaved for 6 hours at 15 to 18 pounds pressure.

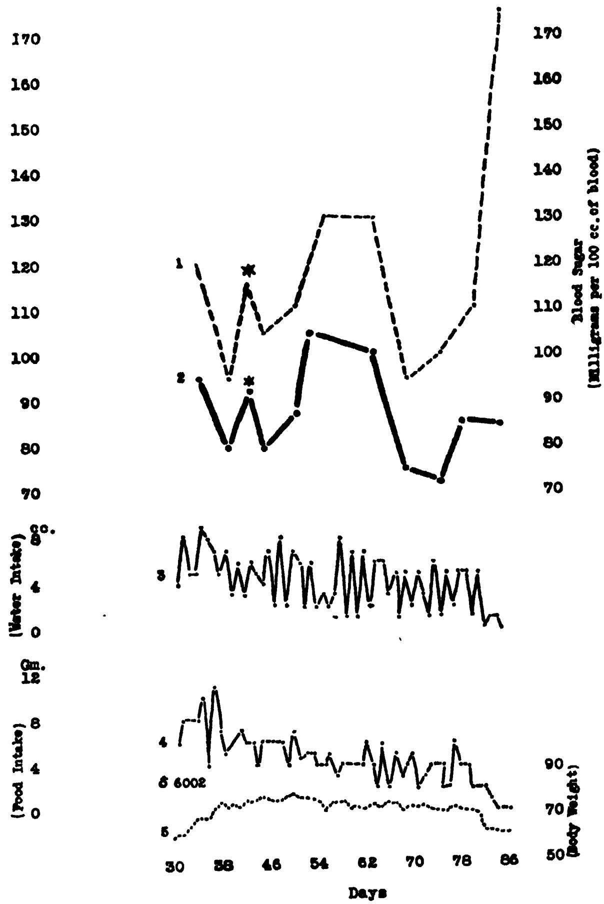

CHART III. The concentration of apparent and true blood sugar in an animal suffering from uncomplicated vitamin B deficiency, 30 to 86 days of age, in a state of prolonged maintenance. During the last 2 days this animal consumed no food and drank only 1 cc. of water. During that period the reducing non-sugars reached a concentration of 90 mg. per cent. During the premortal state enough blood was secured from the carotid artery for a plasma carbon dioxide volume determination, which was 27.5 per cent, indicating marked acidosis, undoubtedly caused by fasting. From point * on the peripheral bleedings were made 2 hours after fasting. Curve 1 represents apparent blood sugar in mg. per cent; Curve 2 represents true blood sugar in mg. per cent; Curve 3 represents daily water intake in cc.; Curve 4 represents daily food consumption in gm.; Curve 5 represents body weight in gm.

which takes place in weaned and adult animals as well as in nursing young. The results of this study will appear shortly in a forthcoming publication.

TABLE IV.

Alkaline Reserve of Control Animals on Ration 1202.

Animal No.	Age.	Weight.	CO_2 volume.
	days	*gm.*	*per cent*
♀ 6008	89	170	52 5
♀ 6009	91	146	42.5
♂ 6010	72	158	53.0
♂ 6010	90	186	45 5
♂ 6011	91	223	42.5
♀ 5824	80	162	48 0
♀ 5285	80	173	48.0
♀ 5826	80	131	53.0
♀ 5826	100	138	48.0
♂ 5827	65	147	53.0
♂ 5828	83	200	55 5
♂ 5829	84	221	44.0
♀ 5718	266	174	40.5
♀ 5812	320	195	55.5
♀ 5813	343	171	60.5
♀ 5742	198	186	53.0
♀ 5814	192	171	53.0
♀ 5751	327	220	45.5
♀ 5732	398	223	52 5
♀ 5732	426	218	58.0
♀ 5756	206	258	45 5
♀ 5756	235	252	60.5
♀ 5757	225	193	50.5
♀ 5757	238	193	53 0
♀ 5765	264	215	50.5
♀ 5765	294	208	55 5

Effect of Uncomplicated Vitamin B Deficiency on Alkaline Reserve of Blood of the Albino Rat.[3]

From Table IV it is apparent that the range of plasma carbon dioxide volume in our control animals is 40.5 to 60.5 per cent, and from Table V that the range in control animals after a 24 hour fast

[3] Credit is due Dr. M. C. Kik for carrying out some of these determinations.

is from 37.5 to 55.5 per cent. With these figures as a basis of comparison, it is clear from Table VI that sixteen out of twenty-three pathological animals studied showed evidences of acidosis. Of these, six gave a reading below 30 volumes per cent, indicating marked acidosis. We associate the acidosis with prolonged inanition rather than with starvation, as manifested by our daily food consumption records.

TABLE V.

Alkaline Reserve of Control Animals on Ration 1202 after a 24 Hour Fasting Period.

Animal No.	Age.	Weight.	Loss of weight.	CO_2 volume.
	days	*gm.*	*gm.*	*per cent*
♀ 6008	69	127	10	40.0
♀ 6009	73	136	10	52.5
♂ 6010	88	171	10	55.5
♂ 6011	85	208	15	48.0
♂ 6012	90	256	15	46.5
♀ 6013	90	134	9	52.5
♀ 5718	297	162	9	50.5
♀ 5813	372	154	9	45.5
♀ 5732	445	216	12	35.5
♀ 5756	265	247	9	42.5
♀ 5757	281	178	10	49.5
♀ 5757	297	189	13	37.5

Effect of Uncomplicated Vitamin B Deficiency in the Albino Rat on Daily Water Intake.

In order to obtain more information on the etiology of anhydremia associated with vitamin B deficiency (7), we conducted detailed experiments on the daily water intake in relation to the daily food ingested. For this purpose, we employed an especially constructed drinking tube of 100 cc. capacity, marked in 1 cc. graduations.[4] Space does not permit us to show all our detailed daily records on thirty-six animals. We are, therefore, summarizing all of our results in Table VII. An examination of Table VII discloses that the polyneuritic animals drank less water than the control animals, which, of course, is to be expected because of lack

[4] Manufactured by Eimer and Amend.

TABLE VI.

Alkaline Reserve of Animals Suffering from Uncomplicated Vitamin B Deficiency.

Animal No.	Age.	Weight.	Change of weight.	CO_2 volume.	Remarks.
	days	*gm.*	*gm.*	*per cent*	
♂ 6002	85	60	−7	25 5	Moribund state; anhydremic. Reducing non-sugars, 90 mg. per cent.
♂ 6003	63	43	−10	31 5	Incipient posterior paralysis; incontinence; anemic; chromogenic urine. Necropsy: blood in ureters; congested lung; hypertrophied heart.
♀ 6004	93	62	+11	45 5	No external gross pathological changes.
♀ 6005	93	51	−8	25 0	Posterior paralysis.
♂ 6006	86	47	−15	28 0	" " anemia. Hemoglobin, 6.7 gm. per 100 cc. blood.
♂ 6007	91	61	+6	38 5	No external gross pathological changes; inanition.
♂ 5801	103	62	+7	35 0	Posterior paralysis; marked inanition. Average daily food intake last 7 days 2 gm. Reducing non-sugars, 81 mg. per cent.
♂ 5803	96	52	−5	27 5	Marked inanition. Average daily food intake last 20 days 1.7 gm. Necropsy: marked intracranial congestion.
♀ 5804	76	44	−5	32 5	Posterior paralysis; pallor of ears; cyanosis of posterior extremities; intracranial congestion.
♀ 5806	91	41	−7	35 0	Asthenia due to marked inanition.
♀ 5816	93	52	−30	38 5	Moribund state.
♀ 5817	77	40	−19	32 0	Incipient paralysis; emaciation. On 75th day reducing non-sugars, 49 mg. per cent.
♀ 5818	65	64	−12	30 0	Marked inanition; slight intracranial congestion.
♀ 5819	65	47	−20	33 0	" "
♀ 5701	330	142	−46	32 0	Complete paralysis; reducing non-sugars, 67 mg. per cent; marked intracranial congestion.
♀ 5738	422	133	−65	26 5	Incipient paralysis; asthenia due to marked inanition.
♀ 5704	384	130	−58	46 5	Slight posterior paralysis in right leg; average daily food intake last 10 days 4 gm.

TABLE VI—*Concluded.*

Animal No.	Age.	Weight.	Change of weight.	CO_2 volume.	Remarks.
	days	*gm.*	*gm.*	*per cent*	
♀ 5705	306	129	−85	40.0	Posterior paralysis; average daily food consumption 3 gm. last 10 days.
♀ 5737	417	155	−47	36 0	Incipient paralysis.
♀ 5736	279	96	−70	27.5	Muscular incoordination; diarrhea. Food consumption last 10 days 3 gm. daily. Necropsy: congestion of lungs, heart, and kidneys.
♀ 5741	186	106	−53	32.0	Muscular incoordination; asthenia due to marked inanition.
♀ 5750	320	126	−79	38.0	Inanition.
♀ 5748	346	149	−58	35 0	Incipient paralysis. Food consumption last 10 days 4.7 gm. daily. Reducing non-sugars, 43 mg. per cent.

of growth and hence a difference in surface area. The comparison, therefore, has to be made on the basis of body weight or preferably surface area. In the absence of surface area measurements, we are making our comparison on the basis of 100 gm. of body weight. When all the results are averaged, the pathological animals show a 13 to 18 per cent reduction in the water intake per 100 gm. of body weight compared with the control animals. On the other hand, the animals suffering from vitamin B deficiency drank 8 to 12 per cent more water per gm. of food ingested. These average figures do not tell the whole story. The question arises, what is the water intake in the terminal stages of the avitaminosis, or in the stage of prolonged maintenance? Is the reduction appreciable enough to produce severe disturbances in the water metabolism of the blood? Our results in this connection are quite variable. Certainly, in a number of animals, drinking only 1 to 3 cc. daily for 3 to 7 days, this is true. Other animals, however, while manifesting marked anorexia, show comparatively little reduction in the water intake. Our conclusions are that the reduction in the water intake during uncomplicated vitamin B deficiency is positively a factor contributing to anhydremia, but the peculiar be-

havior of a number of animals in their comparatively greater water than food intake in the premortal state of polyneuritis would suggest that another as yet unidentified factor is also exerting an influence in the production of concentrated blood. Since the completion of our experiments Stucky and Rose (4) have reported a reduction of water intake in the dog suffering from a deficiency of the vitamin B complex.

TABLE VII.

Summary of Data on Influence of Uncomplicated Vitamin B Deficiency on Daily Water Intake.

Ration No.	No. of animals.	Period of experiment.	Daily water intake.	Daily water intake. Per 100 gm. body weight.	Per gm. food ingested.	Remarks.
		days	*cc.*	*cc.*	*cc.*	
1202	6	60	10 2	7 9	1.00	Control animals during postweaned period.
1452	6	44–63	6.2	6.9	1.08	Pathological animals during postweaned period.
1202	12	28–80	11 5	5 6	1 40	Control adult animals.
1452	12	28–63	6 7	4 6	1.60	Pathological adult animals.

SUMMARY.

1. During uncomplicated vitamin B deficiency in weaned and adult albino rats there is encountered a rise in the reducing non-sugars.

2. Hypoglycemia in such polyneuritic animals is apparent only in the terminal stages of the avitaminosis associated with prolonged inanition.

3. Acidosis occurs in uncomplicated vitamin B deficiency in the later stages associated with prolonged inanition.

4. During uncomplicated vitamin B deficiency there is a reduction of the water intake to the extent of 13 to 18 per cent per 100 gm. of body weight. In some animals the reduction is considerably greater and of sufficient magnitude to warrant the production of pronounced anhydremia.

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A TITRATION METHOD FOR BLOOD FAT.

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The basic idea of the method given in this paper is to isolate the fatty acids and titrate them. The blood is extracted with alcohol and ether, the extract saponified, the fatty acids separated, filtered, washed, dissolved in alcohol, and titrated with phenol blue as an indicator.

In the development of the method, technical difficulties in the filtration and washing of the fatty acids were the chief obstacles. These were finally overcome by the technique of filtration through previously heated paper pulp mats in small Gooch crucibles, salt solution being used for washing.

In a series of ten blood samples from normal fasting subjects the fatty acids averaged 294 mg. per 100 cc. All but one ranged between 260 and 333; the exception was 237. One other supposedly normal sample gave a figure of 193 mg. per 100 cc.

It is believed that a titration method offers certain attractions in the way of simplicity of chemical procedure, and probably freedom from interfering factors in unusual blood samples.

In 1925 Stewart and White[1] published a method similar in general principle. The fatty acids, however, were not separated, washed, and titrated directly as in this method. The alcohol-ether extract of the blood was saponified with 5 cc. of 0.1 N NaOH, then an equivalent amount of 0.1 N HCl was added, the mixture boiled to get rid of CO_2, made up to volume, and an aliquot part (one-tenth) titrated with 0.1 N NaOH. The principal objections to this method are: (1) A very slight error in measuring either the NaOH used for saponification or the HCl used for neutralizing

[1] Stewart, C. P., and White, A. C., *Biochem. J.*, **19**, 840 (1925).

makes a very large per cent error in the calculated blood fat. One of Stewart and White's normal blood fat samples of 300 mg. per 100 cc. of blood would have 3 mg. per cc. or 0.3 mg. in the aliquot

TABLE I.

The materials used were Kahlbaum's oleic and palmitic acids, and Baker and Adamson's C.P. stearic acid. Solutions were made in 95 per cent alcohol. The samples were saponified, etc., as in the method for blood, but of course the preliminary extraction with alcohol-ether mixture and filtration were omitted.

Experiment No.	Oleic.	Palmitic.	Stearic.	Cholesterol.	Total fatty acid.	Found.	Error.
	mM	*mM*	*mM*	*mg.*	*mM*	*mM*	*per cent*
1	0.0646	0.0195	0	0	0.0841	0.0838	−0.30
	0.0646	0.0195	0	0	0.0841	0.0845	+0.50
	0.0646	0.0195	0	0	0.0841	0.0850	+1.20
	0.0646	0.0195	0	0	0.0841	0.0877	+4.40
2	0 0646	0.0195	0	10	0.0841	0.0838	−0.30
	0.0646	0.0195	0	10	0.0841	0.0838	−0.30
3	0 0323	0.0097	0	10	0.0420	0.0430	+2.4
	0.0323	0.0097	0	10	0.0420	0.0430	+2.4
4	0.0969	0.0388	0	0	0.1357	0.1290	−4.9
	0.0969	0.0388	0	0	0.1357	0.131	−3.8
5	0.0626	0.0194	0	0	0.0820	0.0843	+2.8
	0.0626	0.0194	0	0	0.0820	0.0808	−1.2
6	0.0969	0	0.030	10	0.1270	0.1350	+6.3
	0.0969	0	0.030	10	0.1270	0.1320	+4.0
	0.0969	0	0.030	10	0.1270	0.1330	+4.7
7	0.0323	0.0101	0	9	0.0424	0.0422	−0.5
	0.0323	0.0101	0	9	0.0424	0.0408	−3.8
	0.0323	0.0101	0	9	0.0424	0.0420	−0.9
	0.0323	0.0101	0	9	0.0424	0.0420	−0.9
	0.0323	0.0101	0	9	0.0424	0.0407	−4.0

taken for final titration. This would take a titration of only 0.011 cc. of 0.1 N NaOH. An error of only 0.1 per cent in measuring either the NaOH used for saponification or the HCl used for neutralizing would make an error of 45 per cent in the blood fat.

(2) In saponification the glass is attacked and sodium silicate formed, which interferes with the titration. (3) CO_2 will be absorbed during titration unless special precautions are taken.

In the author's method the fatty acids are isolated and washed, and the only amounts of alkali which come into quantitative relations are those used in the direct titration of the fatty acids. Solution of the fatty acids in alcohol removes any silica which may have formed. CO_2 is boiled off at a low pH, and special precautions are taken to avoid absorption during titration.

TABLE II.

Check Determinations on Various Normal and Pathological Blood Samples.

Sample No.	Fatty acid.	Average.	Maximum deviation from average.	Sample No.	Fatty acid.	Average.	Maximum deviation from average.
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*		*mg. per 100 cc.*	*mg. per 100 cc.*	*per cent*
1	362			6	270		
	360				270	270	0
	364	362	0 6	7	538		
2	376				543	540	0.6
	372			8	209		
	379				209	209	0
	370	374	1 4	9	1180		
3	360				1225	1203	2.0
	378			10	498		
	367	368	2.8		510	504	1.2
4	242			11	229		
	237	239	1 2		247	238	3.3
5	288						
	274	281	2 5				

Much work was done on known mixtures of oleic, palmitic, and stearic acids. Not more than 4 parts of oleic to 1 of palmitic or stearic can be filtered and washed quantitatively. These pure fatty acid mixtures are much more difficult to deal with than those derived from blood, partly because cholesterol is present in the blood extracts at the time of filtration, which makes a harder precipitate. In a series of various pathological blood samples none has been found that offers as much difficulty in filtration as the artificial mixtures which, nevertheless, give accurate results.

Addition of cholesterol to the artificial mixtures had no effect on the results.

Tables I to III give the results of test analyses with the method.

TABLE III.

Additions of Fatty Acid to Blood.

A mixture of approximately 3 parts of oleic acid to 1 part of palmitic was used, dissolved in 3 parts of 95 per cent alcohol to 1 part of ether. The blood sample was run into the alcohol-ether mixture used for extraction, and then a measured amount of fatty acid mixture was added. This procedure was adopted to avoid precipitation of proteins by a direct addition of the alcohol-ether solution to blood.

Figures are in mg. of fatty acid per 100 cc. of blood.

Experiment No.	Blood sample.	Average.	Maximum deviation from average.	Blood with added fatty acid.		Average.	Error.
				Calculated.	Found.		
			per cent				*per cent*
1	398.6 384.6			510 6	495.2 498.3	496.7	−2.8
	384.6			867.6	887.6 886.0	886.8	+2.1
	384 6	391.6	1.7	1225.6	1220.9 1231.8	1226.3	+0.03
2	290			409	406 409	407.5	−0.4
				766	756 758	757	−1.2
				1124	1104 1107	1105.5	−1.8
3	265 272	268.5	1.4	387.5	394 406 403	401	+3.4
				744.5	760 759 770	763	+2.5
				1101.5	1103 1133	1118	+1.7

Procedure.

Extraction and Saponification.—5 cc. of whole blood or of plasma or serum are measured into a 100 cc. flask containing about 75 cc. of a mixture of 3 parts of 95 per cent alcohol and 1 part of ethyl ether (redistilled). The blood or plasma is made to enter in a slow stream of drops and the liquid in the flask is kept rotating rapidly to prevent the formation of large aggregates of precipitate. At once, or after waiting until a convenient time, the flask is immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, then cooled to room temperature, made up to volume, mixed by pouring back and forth three times into a dry 300 to 500 cc. flask or beaker with stirring, and filtered through a fat-free filter paper.

Evaporate 75 cc. of the filtrate in a 100 cc. beaker (put in 50 cc. at first). Add a few grains of coarse sand (previously boiled with acid, and then washed, dried, and extracted with ether) to prevent bumping. Place on an electric stove at low heat, or on the cover of a steam bath, or elsewhere, so that it does not boil perceptibly, and allow to evaporate until the ether is practically gone; then place in a gentle current of steam, and continue evaporation until a volume of about 30 cc. is reached. Then add 0.1 cc. of saturated NaOH,[2] mix, add a few grains more of sand, cover with a watch-glass, and boil gently for 20 to 30 minutes (to saponify). Then remove the cover-glass, drop in a small piece of litmus paper, make acid with 30 per cent HCl; then run back to alkalinity with 10 per cent NaOH (in order to avoid an excess of alkali on evaporating to dryness). Evaporate to dryness (in order to get rid of all alcohol). Add 15 cc. of water, heat on the steam bath, and stir to dissolve the soaps. While hot, add a drop of thymol blue indicator and make acid until a faint pink with 30 per cent HCl. Set the beaker for 10 minutes in cold water, then swirl almost continuously for 5 minutes to produce a better separation of the fatty acids.

The filters, which should have been prepared beforehand, are made as follows: Use a Gooch crucible, smallest size (top 28 mm.,

[2] Make a saturated solution of C.P. sodium hydroxide sticks; allow to settle. Make in Nonsol glass if possible, if not, in Pyrex, keeping the flask cool during solution.

bottom 18 mm. in diameter). Set the crucible in a rubber washer which fits over the top of a small funnel. Push the stem of the funnel through the hole in a rubber stopper and set the stopper in a suction flask (500 cc. capcity). Have ready a paper pulp emulsion, made by shaking up a piece of soft filter paper (such as Schleicher and Schüll black ribbon, No. 589) in 300 to 400 cc. of distilled water. Shake this emulsion vigorously and immediately pour some into the crucible while there is a strong suction on. Repeat until a layer about 1 mm. thick is formed. Tamp the layer down carefully all over with the end of a glass rod. Allow the larger masses of filter pulp fibres in the emulsion to settle out, and pour on successive amounts of the thin upper suspension of isolated shreds, keeping a strong suction on and tamping down occasionally, until the filter is dense enough to offer a definite resistance to the suction. Take the crucible out of the rubber washer and dry in an air oven at 110° for 15 minutes. Take out and allow to cool before using.

Set up a row of the funnels which fit the crucible washers. Place a crucible in its washer in a funnel. Place under the funnel a test-tube, and pour into the crucible some of the fatty acid suspension. If the filtrate is not perfectly clear, put it through the crucible again. If the filtration does not start in a few minutes, change the crucible to the funnel in the filter flask and start the suction very gently, with a test-tube under the funnel. After filtration has started, continue without suction. After the fatty acid suspension is filtered and has drained, wash with 4 cc. of 5 per cent NaCl solution, which has been neutralized to methyl red. Use a pipette and run the salt solution down the walls of the beaker all around, then, tipping the beaker, use a fine bent glass rod to rinse the side of the beaker more thoroughly with the solution, then pour this rinsing into the Gooch crucible, rinsing its side with the aid of the rod. Wash until the filtrate from one washing takes not over 0.05 cc. of 0.02 N NaOH to neutralize it to phenolphthalein. Usually this is true of the third washing.

Put the crucible back on the suction flask with a non-protein nitrogen tube (cut off to a convenient height and calibrated at 1 cc. intervals from 10 to 15 cc.) under the funnel. Wash down the walls of the beaker with 5 cc. of 95 per cent alcohol, heat to

boiling, and pour into the crucible. With the glass rod quickly loosen up any fatty acid fragments on the wall of the crucible. Allow to run nearly out, then put on a moderate suction. Rinse out the beaker and crucible twice more with 3 cc. of alcohol each time, heating it to boiling. Then wash off the outside of the crucible and the funnel.

Titration.—Add a few grains of sand, boil the filtrate for 1 minute, cool in a beaker of water, note the volume of alcohol, add 3 drops of 0.3 per cent ~~phenol~~ blue in 50 per cent alcohol, titrate with 0.02 N NaOH to a pure blue which stays practically unchanged with no yellow tinge while stirring it for 2 minutes with a stopper in the mouth of the tube to avoid absorption of CO_2.

For a blank boil 10 cc. of alcohol and titrate. Calculate the correction necessary for the amount of alcohol present before titration. Add a correction amounting to 0.005 cc. for each cc. of NaOH used in titration (a simple correction for the volume of solution). Subtract the total correction from the titration. Multiply by the normality factor, thus getting the number of millimols of fatty acid. Multiply by

$$\frac{100}{\text{cc. filtrate evaporated}} \times \frac{100}{\text{cc. blood used}}$$

millimols of fatty acid per 100 cc. of blood. To translate into terms of weight (not a very significant figure) multiply by an average factor for the fatty acids as they usually occur in blood = 277.2. The molecular weights are so nearly alike that a considerable variation in the proportions will not affect the calculated weight more than about 2 per cent.

Note that cholesterol is present while the fatty acids are titrated. The cholesterol does not affect the titration.

Determination of Cholesterol.—Transfer the titrated fatty acids to a 100 cc. beaker, make acid with 3 N HCl, add a few grains of sand to prevent bumping, and evaporate to dryness on the steam bath. Add 10 cc. of chloroform, stir to dissolve, allow to stand 10 minutes, filter through a paper pulp filter in a Gooch crucible into a 20 cc. volumetric flask. Extract twice more with 5 cc. of chloroform for 5 minutes each time. Make the flask up to volume with chloroform, mix by pouring back and forth three times into a flask, then

take 2 cc. for cholesterol determination according to Bloor's method by the Liebermann-Burchard reaction.

Note that the fatty acids are present while the cholesterol is being determined. It was found that they give no color reaction and do not interfere with the color reaction given by cholesterol.

This paper is the first in a series on fat metabolism, projected by Dr. Chester M. Jones and the authors.

THE REACTION OF BORATE AND SUGARS.

II. THE OPTICAL ACTIVITY OF SUGARS IN BORAX SOLUTION AND THE CONFIGURATION OF MUTAROTATORY ISOMERS.*

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(Received for publication, August 26, 1929.)

During our study of the effect of borate on the oxidation of sugars (Levy and Doisy, 1928), our attention was attracted to the investigations of the effect of borax on the optical activity of glucose and fructose apparently first reported by Rimbach and Weber (1905). They observed that the optical activities of these sugars were less (*i.e.* nearer 0°) in borax solutions than in distilled water but that the values of the optical activities were normal in boric acid solutions. They advanced the hypothesis that their observations could be explained adequately by the known effect of alkali upon carbohydrates and by formation of a compound between the borax and sugar.

Murchhauser (1923) observed the decreased rotatory power of glucose in the presence of borax and stated that this decrease was a specific effect of borax and indicated that probably the decrease had nothing to do with the alkalinity. He was impressed by the constancy of the final rotation at a much higher value than would be expected even with weakly alkaline solutions. This exceptional behavior had also been observed by Rimbach and Weber (1905) but the latter believed that this merely showed that the solution had become neutral through the formation of saccharinic acids

* This paper and the previous one of this series (Levy and Doisy, 1928) are based on data taken from the Dissertation submitted by Milton Levy to the Graduate School of St. Louis University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

from the sugar. Michaelis and Rona (1912) have also observed the decreased rotation of glucose in borax solution.

Glucose (Boeseken, 1913), galactose and fructose (Boeseken, Kerstjens, and Klamer, 1916), xylose, arabinose, rhamnose, and mannose (Boeseken and Couvert, 1921) increase the conductivity of boric acid, and the α- and β-isomers of each sugar are effective to different degrees. A consideration of the type of compound which Boeseken's systematic investigations have shown to be effective in increasing the conductivity of boric acid has led this author to assign to the α and β forms of each sugar a definite configuration for the end aldehydic carbon atom.

The present experimental work began with observations made during the course of work on the effect of borate on the oxidation of sugars (Levy and Doisy, 1928) when it was shown that the effect of borax on the optical activity of glucose was reversible in the sense that the activity of the equilibrated α, β-mixture could be restored by simply adding sufficient acid to convert the borax to a sodium salt and boric acid. The changes of the optical activity on acidification of the borax solution were not simple but indicated the liberation of an excess of α-glucose which then mutarotated to the normal value. This appeared to be a direct confirmation of Boeseken's deduction from conductivity measurements that α-glucose is more favorably oriented than β-glucose for complex formation with boric acid. The measurement of the optical activities of sugars and certain derivatives in borax solution and the effect of acidification constitute the experimental basis of the present paper. The experimental data are discussed with respect to the formulation of the α, β-isomers of the sugars.

Methods.

In general the mutarotation and optical activity of the sugar in 0.5 M solution were followed in distilled water, in 0.2 M boric acid, and in 0.05 M borax (0.1 M NaOH + 0.2 M H_3BO_3 (Kahlenberg and Schreiner, 1896)). After equilibrium of the last named solution had been attained, it was mixed with an equal volume of 0.1 N or usually 0.11 N HCl and the rotatory changes followed. A Schmidt and Haensch polarimeter reading to 0.01° and a special sodium lamp designed by West (1928) were used. No attempt was made to regulate the temperature of the solutions but the

dark room remained quite constant in temperature during the day and the variations from day to day were not great.

The method of operation was to weigh the sugar into a beaker and if either boric acid or borax was to be used, it was dissolved in the water. 10 seconds before zero time, the solvent was poured over the sugar, and stirred rapidly until solution occurred. The solution was transferred to a volumetric flask which was filled to the mark and its contents thoroughly mixed. The polarimeter tube (2 dm. tubes were used throughout) which had been previously dried was immediately filled and readings started. With practice it was found possible to obtain readings in from 2 to 5 minutes, depending on the rate of solution of the sugar used. In the acidification experiments, 10 cc. portions of the acid and 10 cc. of the equilibrated sugar borax solution were pipetted into separate beakers. At zero time, the two solutions were poured back and forth until completely mixed and the mixture was then placed in the polarimeter tube. Readings were started as soon as possible but usually 1 or 2 minutes had elapsed before the first reading could be completed. The usual precautions were observed in reading the polarimeter.

Boric acid was prepared from a c.p. sample by three crystallizations from water, followed by drying over $CaCl_2$. By titration in the presence of excess glucose (Weatherby and Chesny, 1926) it utilized 99.8 per cent of the theoretical quantity of NaOH.

The samples of α- and β-glucose were prepared according to the directions of Hudson and Dale (1917). The α-glucose gave an initial $[\alpha]_D$ of 109° and the β-glucose of 20°. The final rotations were 52.5° and 52.3° respectively.

α-Methylglucoside was prepared according to the method given by Helferich and Schäfer (1926). It melted at 167° and showed $[\alpha]_D = 158.1°$.

2,3,4,6-Tetramethylglucose was prepared by the methylation of glucose and subsequent hydrolysis of the methyltetramethylglucoside, according to Haworth (1915). The sample melted at 82–84° and showed $[\alpha]_D = 79.4°$.

Mannose was recrystallized from acetic acid according to Levene (1924). Its behavior corresponded to β-mannose rather than α-mannose, which is supposed to be the result of this method. The final value of $[\alpha]_D$ was 14°.

The remainder of the sugars were high grade commercial samples such as were used in our earlier work.

EXPERIMENTS AND DISCUSSION.

In Fig. 1 are shown the mutarotation curves for α- and β-glucose in water and boric acid solution (Curves I and III) and in 0.05 M

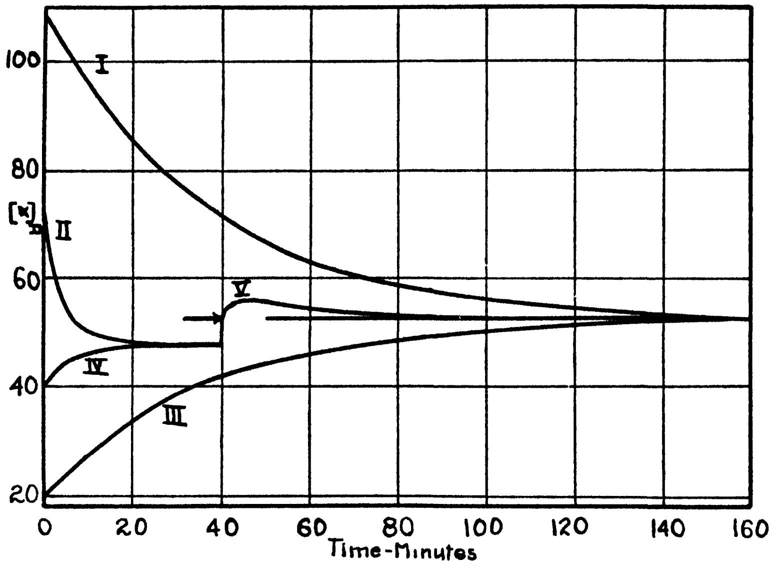


FIG. 1. Rotatory changes of α- and β-glucose. Curve I, mutarotation of 0.5 M α-glucose in distilled water or 0.2 M H_3BO_3. Curve II, 0.5 M α-glucose in 0.05 M borax. Curve III, 0.5 M β-glucose in distilled water or 0.2 M H_3BO_3. Curve IV, 0.5 M β-glucose in 0.05 M borax. The arrow indicates the specific rotation of α,β-glucose and zero time for the curve (Curve V) obtained on acidification. The borax solution was actually acidified 24 hours after preparation.

$Na_2B_4O_7$ (Curves II and IV). The values of the specific rotation at zero time were obtained by extrapolation of the logarithmic curve of the monomolecular reaction in the usual way. It may be noted that the data upon which the curves are based conform to the monomolecular law very well, even in the borax solutions. At equilibrium glucose (0.5 M) shows $[\alpha]_D = 52.5°$ in water or boric acid and $[\alpha]_D = 48°$ in 0.05 M borax.

The fact that the glucose-borax mixtures are acid to litmus immediately after preparation excludes any change in $[\alpha]_D$ due to alkalinity, and the truth of the statement that no irreversible changes occur is shown by the behavior of the optical activity on addition of acid to the borax solutions. This addition was made 24 hours after the preparation of the borax-glucose solution but this time has been conveniently shortened on the graph. On the addition of acid the optical activity of the glucose rose *above* the equilibrium value and then slowly fell to the value found with glucose in water or boric acid solution. It made no difference whether the solution acidified was originally prepared with α-glucose or β-glucose; the resulting curve was the same.

Our interpretation is that in the borax solution there is present the sodium salt of a complex acid formed by the combination of glucose and boric acid. The glucose in this form has a lower optical activity than when free. On acidification this complex acid is liberated and its hydrolysis to a mixture containing an excess of α-glucose causes the initial rise in activity. The fall to the normal equilibrium value is due to the mutarotation of the α-glucose so liberated.

The effect of the addition of acid is shown on a larger scale in Fig. 2. In this experiment 0.1 M borax was used and a 10 per cent excess of acid was added. The extra acid hastens the hydrolysis of the free acid complex and the curve of decreasing rotation is shown in Table I to follow the monomolecular law giving a constant equal to 0.014 ± 0.001 (based on common logarithms) after the 5th minute (Table I). This is the type of curve expected if an excess of α-glucose had been liberated on hydrolysis of the complex.

Consideration of the acidity of the borax-glucose solutions shows that practically all of the NaOH in the solution of Fig. 1 is neutralized by the complex acid rather than by boric acid. This solution is acid to litmus. We may assume that the solution has a pH of 7. For the boric acid we may set up Equation 1.

$$7 = 9.2 + \log \frac{[BO_2^-]}{[HBO_2]} \tag{1}$$

in which 9.2 is the value of pK_a for boric acid given by Clark (1928).

Solving, we find that $\frac{[BO_2^-]}{[HBO_2]}$ is 0.006. $[HBO_2] = 0.1$ M; consequently $[BO_2^-]$ must be of the order of 0.0006 M; of the total of the 0.1 M Na^+ present, practically all is neutralized by the complex acid. In the following paper (Levy, 1929) it will be shown by means of freezing point measurements that the amount of glucose

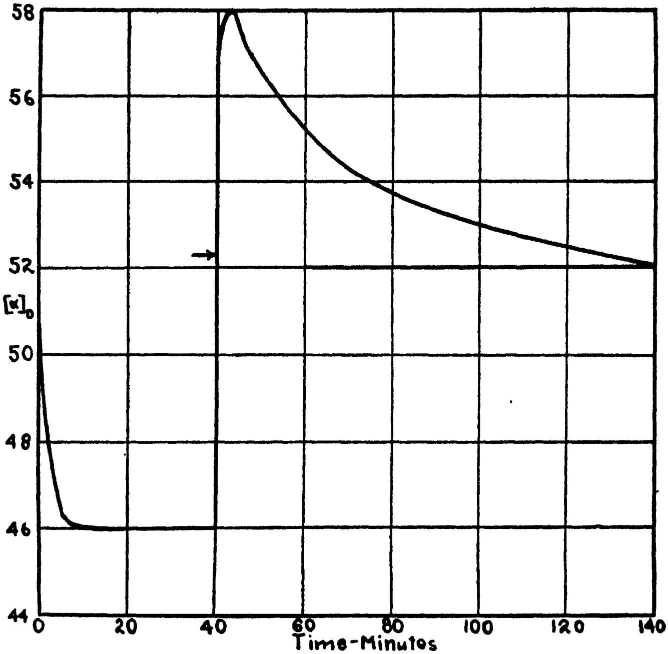


FIG. 2. Rotatory changes of α-glucose in borax and the effect of acidification. α-glucose 0.5 M, borax 0.1 M. The arrow indicates zero time for the addition of excess acid to the borax solution which actually occurred 24 hours after the preparation of the original solution. It also indicates the specific rotation of α, β-glucose in distilled water or boric acid.

combined is equal to the $[Na^+]$ of these solutions, or 0.1 M. It is this combined glucose which is responsible for the changes of optical activity.

The interpretation of the equilibrium rotation of glucose as being simply due to a balanced reaction between α- and β-glucose, perhaps through an intermediary present in low concentration, has

been shown experimentally to be somewhat inadequate (Lowry, 1927). However, on the basis of the older ideas, the increase in optical activity of our glucose-borax solutions on acidification may be interpreted in terms of actual amounts of α- and β-glucose liberated. With the total amount of glucose liberated from the complex taken to be 0.1 M, the maximum activity obtained after acidification corresponded in various experiments to the liberation of a mixture containing between 55 and 66 per cent of α-glucose and 45 and 34 per cent of β-glucose. In these experiments the latter parts of the curves obtained on acidification were extra-

TABLE I.

Mutarotation of α-Glucose Liberated on Acidification of Glucose-Borax Solution.

Glucose 0.5 M, NaOH 0.2 M, H_3BO_3 0.4 M + equal volume of 0.22 N HCl.

Time after acidification.	α, 2 dm. tube.	k* for mutarotation.
min.	*degrees*	
1	5.18	
2	5.22	
3	5.22	0.000
4	5 16	0.025
5	5 17	0 0140
10	5 10	0.0135
20	4 97	0.0146
30	4 86	0.0149
40	4 83	0.0141
Final.	4 674	

* Calculated from 5.22° as the value of α_0 at 2 minutes after acidification and on the basis of common logs.

polated to zero time if the values gave a satisfactory constant, but the maximum rotation was used if it could not be shown that the final change followed the monomolecular law. The extrapolated values gave the higher percentage of α-glucose. These figures are considered direct evidence that a complex is formed between α-glucose and borate to a greater extent than between β-glucose and borate (or, the existence of only one complex being assumed, that the configuration of glucose in this complex differs less from the configuration of α-glucose than from β-glucose).

The fact that the α- and β-isomers of glucose behave differently

towards boric acid as measured by their respective influences on the conductivity (Boeseken, 1913), as well as the experiments just described, and the interference of borate with the oxidation of the aldehyde group (Levy and Doisy, 1928) are evidences that the aldehydic hydroxyl group of the mutarotatory isomers is involved in the complex formation with borate. This is further confirmed by the fact that blocking this hydroxyl group as in α- and β-methylglucosides eliminates the effect on the conductivity (Boeseken and Couvert, 1921), and by the experiments of Table II which show that borate has only a slight effect on the specific rotation of α-methylglucoside.

While Table II shows a slight decrease in the optical activity of α-methylglucoside in the presence of borate, none of the changes characteristic of glucose occur following acidification, the rotation

TABLE II.

Effect of Borax on the Rotatory Power of α-Methylglucoside.

The glucoside was 0 5 M in each solution except Solution 4.

Solution No.	Solvent.	α 2 dm tube	$[\alpha]_D$
		degrees	*degrees*
1	H_2O	30 692	158 08
2	0 2 M H_3BO_3.	30 696	158 10
3	0 05 M $Na_2B_4O_7$.	30 361	156 32
4	10 cc. Solution 3 + 10 cc 0.1 N HCl.	15 361	158 24

rising immediately to the final value given. This leads to the conclusion that these changes with glucose must have been dependent on the presence of the free aldehydic hydroxyl group.

Boeseken (1913) from his systematic studies of the effect of various hydroxyl-containing compounds on the conductivity of boric acid has concluded that complex formation occurs with such a compound when two hydroxyl groups are situtated on adjacent carbon atoms and on the same side of the plane in which these carbon atoms lie. If the aldehydic hydroxyl group is involved in the complex formation, it follows from this generalization that the hydroxyl group of carbon atom 2 is also involved. This is the basic principle underlying the formulæ for α- and β-isomers suggested by Boeseken for glucose and other sugars.

If Boeseken's principal assumption is correct, 2,3,4,6-tetra-

methylglucose should not combine with boric acid because the hydroxyl group of carbon atom 2 is blocked. Irvine and Steele (1915) tested the substance and concluded that the methylated glucose increased the conductivity of boric acid quite as much as did glucose and that the conductivity changed in the same manner as the rotation during mutarotation. However, Boeseken and Couvert (1921) repeated these experiments in exactly the same way and their results show that tetramethylglucose does not combine with boric acid, so that, in view of our studies which show no evidence of combination between the methylated sugar and borax, the apparent contradiction of Irvine and Steele's data is probably due to an experimental factor.

TABLE III.

Rate of Oxidation of Tetramethylglucose by Alkaline Iodine Solution.

Time.	Per cent oxidation.	
	Na_2CO_3, $NaHCO_3$ pH = 10	Na_2CO_3, H_3BO_3 pH = 10 1
min.		
2	55 2	64 7
5	76 2	81 8
10	88 2	93 7
20	98 2	99 3
40	100 2	100.3
60	100 5	100 5
120	100 7	100.7

Table III gives data on the oxidation of tetramethylglucose by alkaline iodine solutions, the experiments being performed in the manner described by Levy and Doisy (1928). The slightly greater rate of oxidation in the borate-carbonate buffer is due to the slightly greater alkalinity as indicated by the pH of the buffer. The difference is perhaps exaggerated because of the unavoidable experimental error in following such a rapid reaction. Since these data show that borate does not interfere with the oxidation, it is improbable that a complex is formed.

In addition to the evidence in Table III, the optical activity of the methylated sugar is but slightly affected by borax under the conditions which gave a marked change in the optical activity of

glucose and the solution shows very little, if any, of the changes on acidification characteristic of glucose (Table IV).

The evidence so far submitted indicates that the glucosidic hydroxyl group and one of the chain hydroxyl groups are involved in complex formation. The particular chain hydroxyl group involved is probably the one adjacent to the aldehyde group (carbon atom 2). The results of acidification show that α-glucose is the more favorably oriented of the two forms and should be given the *cis* formulation in the ring formulations of sugars adopted by Boeseken (1913) to bring out this relationship, and more recently advocated by Goodyear and Haworth (1927) and by Haworth (1929) to show the relationship of the sugars to pyran and furan. If we adopt Haworth's formulations of glucose as a derivative of

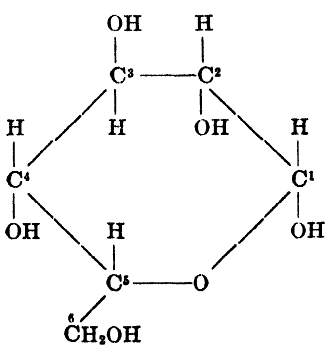
TABLE IV.

Effect of Borax on the Optical Activity of 2,3,4,6-Tetramethylglucose.

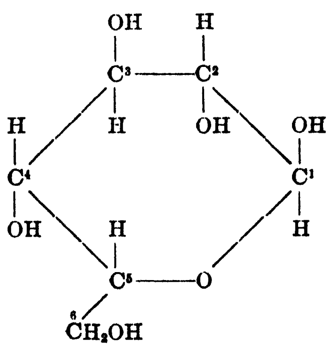
The concentration of tetramethylglucose was 0.5 M except in Solution 4.

Solution No.	Solvent.	Initial.		Final.		k for mutarotation.
		α	$[\alpha]_D$	α	$[\alpha]_D$	
		degrees	*degrees*	*degrees*	*degrees*	
1	H_2O	21.89	92.67	18.76	79.42	0.013
2	0.2 M H_3BO_3.	21.83	92.42	18.75	79.36	0.014
3	0.05 M $Na_2B_4O_7$.	20.09	85.05	18.57	78.63	0.18
4	10 cc. Solution 3 + 10 cc. 0.1 N HCl.	9 33	78.83	9.37	79.33	

pyran, α-glucose is represented by Formula I and β-glucose by Formula II. These are the formulæ adopted by Boeseken (1913)



I. α-Glucose.



II. β-Glucose.

and correspond to those advocated by Pictet (1920) and Michaelis (1913) for these isomers from entirely independent evidence.

Hudson (1909) has shown that the α- and β-isomers of a sugar represent the two possible configurations of carbon atom 1 as an asymmetric center. If the formulæ deduced above are correct, then in α-glucose carbon atom 1 has the same configuration as carbon atom 2, which is *d* (Wohl and Freudenberg, 1923), and in β-glucose the opposite, or *l*. According to Hudson's nomenclature all α-aldose sugars of the *d* series (genetically related to *d*-glucose) will have the same configuration of carbon atom 1, or the *d* configuration according to the above formulæ, and all β-aldose sugars of this series the opposite or *l* configuration. Similarly, all α-aldoses of the *l* series will have the same configuration of carbon atom 1, which will be opposite to that of the α-sugars of the *d* series, or, according to the above formulæ, the *l* configuration, and the β-sugars of the *l* series will have the *d* configuration of carbon atom 1. These requirements are fully met by the conclusions drawn from experiments on the optical activity of various sugars in borax and the effect of acidification which will be presented below.

The curves of Fig. 3 represent the mutarotation of the various sugars in borax solution and the effect of acidification. In some cases (lactose and maltose) mutarotation was so rapid that it could not be followed accurately and in these cases the mutarotation curve is omitted. It is noteworthy that these solutions were quite alkaline and freezing point data show a lesser extent of complex formation than with other sugars. The main point of interest, however, is the behavior of the optical activities on acidification. In every case except mannose, the more dextrorotatory form is liberated in excess of the equilibrium normally expected. The case of rhamnose is not particularly marked, although the data obtained do show the liberation of the dextro form. With mannose the more levorotatory isomer is liberated on acidification. The stereochemical formulation of each aldose used has for carbon atom 2 the *d* configuration except mannose, in which carbon atom 2 has the *l* configuration. In the form which combines most readily with borate, as shown by its liberation when the complex is decomposed, carbon atoms 1 and 2 are presumed to have the same configuration. Consequently the more dextrorotatory form of the

pair of isomers for each sugar has the *d* configuration for carbon atom 1. This relation is made apparent in Table V. Fructose is not included since it is a keto sugar and the principle of optical superposition precludes the application of relationships determined

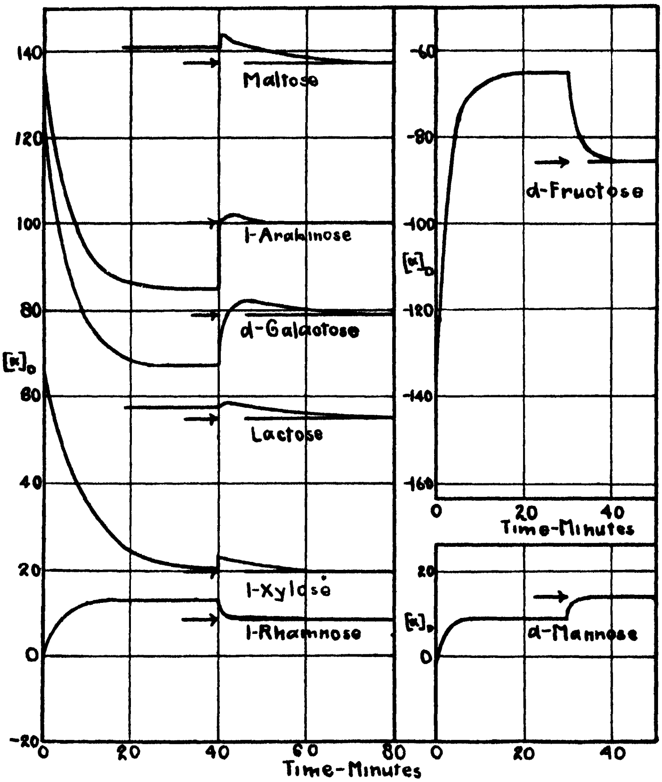


FIG. 3. Rotatory changes of various sugars in borax and the effect of acidification. The sugar in each case is 0.5 M and the borax 0.05 M. The arrows indicate zero time for the acidification (24 hours after preparation of the sugar-borax solution) and the specific rotation of the sugar in distilled water or 0.2 M boric acid at equilibrium.

for the aldoses (Hudson, 1909). In addition complex formation could occur between carbon atoms 1 and 2 or 2 and 3 just as with the acetone derivatives of fructose (Anderson, Charlton, Haworth, and Nicholson, 1929) and one cannot be sure what configurational

significance the fact that the dextrorotatory form is liberated on acidification may have.

The configurations given in Table V agree with those adopted by Boeseken (1913), Boeseken, Kerstjens, and Klamer (1916), and Boeseken and Couvert (1921) from conductivity data, except for mannose. With this sugar the latter authors found that the conductivity increases as the α-isomer is formed. It seems to the present writers that the method described in this paper is subject to less disturbing influence. The conductivity of the solutions of sugars and boric acid depends on two factors: (1) The amount of complex acid formed which is the criterion by which the stereochemical relations should be judged, and (2) the dissociation of the acid formed, which is not necessarily related to the configuration of carbon atom 1. The increase in conductivity is a measure of both factors, whereas the optical method described here depends only on the amount of complex acid formed. It is felt therefore that the configuration presented for mannose is more reliable than Boeseken's configuration. In addition the present configuration is consistent with Hudson's (1909) generalizations.

TABLE V.

Configuration of Carbon Atom 1 of Aldoses.

Sugar	$[\alpha]_D$ of form liberated in excess.	$[\alpha]_D$ of other form.	Configuration.	
			Carbon atom 2.	Dextro form of carbon atom 1
	degrees	*degrees*		
d-Glucose	109	20	*d*	*d*
d-Mannose	−17	34	*l*	"
d-Galactose	140	53	*d*	"
l-Arabinose	184	76	"	"
l-Xylose	92	−20	"	"
l-Rhamnose	54	−7.7	"	"
Maltose	168	11 8	"	"
Lactose	86	35 0	"	"

CONCLUSIONS.

1. The optical activities of sugars differ in borax solution from the optical activities in water.

2. The expected activity is restored when the base of the borax is neutralized by a strong acid.

3. The acidified solutions undergo rotatory changes before reaching equilibrium, which can be correlated with the configuration of carbon atoms 1 and 2 of the aldehyde sugars.

4. The data obtained have been used to show that the more dextrorotatory of the α- and β-isomers of each aldose sugar has the *d* configuration for the aldehydic carbon atom.

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THE REACTION OF BORATE AND SUGARS.

III. THE FREEZING POINT LOWERING OF SUGARS IN BORAX SOLUTIONS.*

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The interpretation of our data on the optical activities of sugars in borax solution and the effects attending the acidification of these solutions (Levy and Doisy, 1929) required a knowledge of the amount of sugar combined in the solution. Three possible methods may be outlined. The most direct would be to prepare in a pure state the compounds of the various sugars and borate and establish by analysis their composition, and from some property, perhaps the optical activity, establish a method for the determination of the reaction product in our equilibrated solutions. However, we have not been able to isolate such compounds.

A second method depending on the determination of the equilibrium in the solution between boric acid and its salts, as determined by the pH of the solutions, has been outlined in the preceding paper. This method really determines the amount of borate ion entering into complex formation and not the amount of sugar. Only by assuming the composition of the compound could this be related to the amount of sugar combined. Since it was not possible to isolate the compounds, it would be necessary here to reason by analogy. Hermans (1925) has stated that the complex formed with boric acid and hydroxy compounds contains 2 molecules of hydroxy compound per molecule of boric acid if the

* This paper is an extension of certain experiments contained in the Dissertation submitted by Milton Levy to the Graduate School of St. Louis University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

conductivity of boric acid is increased, and only 1 molecule if it is not increased. The distinction between the two classes of compounds on the basis of conductivity seems to call for some comment. Theoretically, a complex formed between 2 molecules of hydroxy compound and 1 molecule of boric acid should be a stronger acid than the boric acid, as has been shown by Hermans. It is not necessarily true, however, that a compound of the 1:1 type will be weaker than boric acid or of the same strength. The dissociation of the acid hydrogen would be influenced by the nature of the hydroxy component entering the complex, and the possibility that the complex could be dissociated to a greater extent than boric acid and so cause an increase in conductivity is not excluded. It appears then that the second method of study is uncertain with regard to the amount of sugar combined because the composition of the complex is unknown.

The third possible method is the study of one of the colligative properties of the solution and the most convenient of these is the freezing point. If no compound formed between borax and the sugar, the freezing point lowering of the mixed solution should be the sum of the freezing point lowerings due to each component. If a complex is formed, the freezing point lowering will be less than the sum, and the difference between the sum and the observed lowering divided by the molecular freezing point lowering should give the concentration of combined sugar; comparison of this value with the amount of borate combined as determined by the second method should establish the composition of the compound.

For the present purpose, a borax solution may be considered as containing equimolecular amounts of $NaBO_2$ and HBO_2 (Kahlenberg and Schreiner, 1896). The freezing point lowering of the borax solution is accounted for by the partial lowerings caused by Na^+, BO_2^-, $NaBO_2$, and HBO_2. The dissociation of HBO_2 can be disregarded, since it is a weak acid in the presence of its salt. Complex formation with HBO_2 is negligible (as a first approximation) as shown by the lack of a measureable change of the optical activities of sugars in its presence. The complex with which we are dealing exists in the solution in the form of its salt and the freezing point lowering of a solution containing borax and sugar may be attributed to the sum of the partial lowerings due to Na^+, BO_2^-, $NaBO_2$, HBO_2, compound ion, undissociated salt of the

compound, and free sugar. Part of the borate ion and sodium borate in the borax solutions has been replaced by the compound ion and undissociated sugar-borate compound. There is no reason for assuming that this compound will be other than monovalent; consequently, neglecting the difference in degree of dissociation of the two salts, we may assume that the partial freezing point lowering due to the $NaBO_2$ entering into complex formation will be replaced by the partial lowering due to the complex and its salt. If we subtract the lowering of borax solution from the observed lowering, the difference will be due to the free sugar, and this divided by the value of the molecular freezing point constant will give the value of the free sugar concentration. The difference between the amount of sugar added and the amount of free sugar is the amount of sugar entering the complex.

The second method can be used to show the ratio of total borate to boric acid and if we neglect any compound formation with the acid, the difference between the original and final $[BO_2^-]$ will be the amount of borate entering into compound formation. A comparison of the results of the two methods will give an insight into the composition of the compound formed.

The difference in the degree of dissociation of the sodium salt of the compound and of sodium borate will disturb the results by the freezing point method. If the complex salt is ionized to a greater extent than $NaBO_2$, then the amount of combination will be greater than that actually calculated from the freezing point data, and if the complex is dissociated to a lesser extent, the complex formation will be less than calculated. This variation should not be greater than 20 per cent, however, since the degrees of dissociation of most salts at the concentrations we have used vary over a range of 0.60 to 0.95.

With the limitations in mind, we may use the data of Kahlenberg and Schreiner (1896) on the freezing points of mannitol-borax solutions to illustrate the method of calculation. They found that Δ_B for 0.1 M borax solution was 0.720° and for a solution containing 0.4 M mannitol in addition to 0.1 M borax $\Delta_S = 1.120°$. The difference between the two $(\Delta_S - \Delta_B)$ is 0.400°. This divided by 1.86° (the molecular freezing point lowering for water) gives 0.215 mols as the amount of free mannitol. The amount combined is then 0.4 − 0.215 = 0.185 mols. Similar calculations

for other concentrations of mannitol from data given by Kahlenberg and Schreiner are shown in Table I.

It is evident from Table I that 0.2 M mannitol enters into compound formation with 0.1 M borax. The solutions are neutral or slightly acid (our observation) consequently no free borate remained in the solution, and since it was originally 0.2 M in borate, the complex in the solution was formed between 1 molecule of $NaBO_2$ and 1 molecule of mannitol, corresponding to the mono-mannito-monoboric acid isolated by Fox and Guage (1911), the existence of which Hermans doubted because he was unable to prepare it and because of theoretical considerations.

TABLE I.

Amount of Mannitol Involved in Complex Formation with Borate.

Data of Kahlenberg and Schreiner.

Borax, *M*	0.1	0.1	0.1	0.1	0.1
Mannitol, *M*	0.2	0.4	0.6	0.8	
Δ, *degrees*	0.792	1.120	1.449	1.845	0.720
$\Delta_S - \Delta_B$, *degrees*	0.072	0.400	0.729	1.125	
Free mannitol, *M*	0.039	0.215	0.392	0.605	
Combined mannitol, *M*	0.161	0.185	0.208	0.195	

Methods.

Freezing points were determined by the usual arrangement except that in order to conserve certain of the materials, a micro-Beckmann thermometer and apparatus suitable for the use of 10 cc. of solution were used. Δ_B of 0.05 M borax solutions (0.2 M H_3BO_3 + 0.1 M NaOH) was determined and the freezing point lowering of the sugar alone in each case as a check. The sugar solutions were prepared by adding 10 cc. of water or 0.05 M borax to the weighed quantity of sugar so that the concentrations were approximately weight molar and disturbances due to displacement of water by the solute in the volume system were avoided.

The sugars and other materials used were described in the preceding paper (Levy and Doisy, 1929).

EXPERIMENTAL AND DISCUSSION.

The results are shown in Table II.

The concentration of combined sugar, which is the significant value in Table II, is shown in the last column. Every solution

TABLE II.

Compound Formation in Borax-Sugar Solutions from Freezing Point Data.

$\Delta_{borax} = 0.426°$. Each solution contained 0.05 M borax.

Sugar.	Concentration.	Δ_s	$\Delta_s - 0.426$	Free sugar.	Combined sugar.
	M	*degrees*	*degrees*	*M*	*M*
d-Glucose.	0.5	1.151	0.725	0.390	0.110
	0.4	0.981	0.555	0.298	0.102
	0.25	0.696	0.270	0.145	0.105
	0.2	0.607	0.181	0.097	0.103
d-Mannose.	0.5	1.171	0.745	0.400	0.100
	0.25	0.745	0.399	0.172	0.078
d-Galactose.	0.5	1.110	0.684	0.368	0.132
	0.25	0.650	0.224	0.121	0.129
d-Fructose.	0.5	1.075	0.649	0.349	0.151
	0.25	0.605	0.179	0.096	0.154
l-Rhamnose.	0.5	1.265	0.839	0.453	0.047
	0.25	0.797	0.371	0.199	0.051
l-Xylose.	0.5	1.103	0.704	0.378	0.122
	0.25	0.680	0.254	0.139	0.111
l-Arabinose.	0.5	1.185	0.759	0.408	0.092
	0.25	0.695	0.269	0.145	0.105
Maltose.	0.5	1.179	0.753	0.405	0.095
	0.25	0.735	0.309	0.166	0.084
Lactose.	0.5	1.264	0.838	0.452	0.048
	0.25	0.777	0.351	0.189	0.061
2,3,4,6-Tetramethylglucose.*	0.5	1.525	1.099	0.592	0.000
	0.25	0.951	0.525	0.284	0.000
Methylglucoside.	0.5	1.362	0.936	0.503	0.000
	0.25	0.864	0.438	0.236	0.014

* This derivative gave an abnormally high Δ in water, possibly because of impurities. This explains the high values of free sugar.

which showed a pH value less than 8.0 gave a value of combined sugar sufficiently close to 0.1 M to justify the conclusion that the compounds which are formed are made up of 1 molecule of sugar and 1 molecule of $NaBO_2$. A pH value of 8 or lower indicates that practically all of the $NaBO_2$ originally present in the borax has been used up in complex formation. It is interesting to note that halving the amount of sugar added had comparatively little, if any, effect on the extent of complex formation. However, in every case the solution to which the greater amount of sugar had been added was the more acid as determined by the behavior of indicators. The increased acidity probably indicated complex formation with boric acid itself. In those cases where the amount of combined sugar was considerably higher than 0.1 M, the solutions showed distinctly lower pH values. Since the solution of fructose (0.5 M) and borax gives a colorimetric pH of 5.5, there can be little doubt that part of the combined sugar (0.15 M) reacts with boric acid, the difference being exaggerated perhaps by experimental error and differences in degrees of dissociation of the complex salt and $NaBO_2$. The other two sugars which show high values for combined sugar (galactose, 0.13 and xylose, 0.12) give pH values of 6.5 in solutions containing 0.5 M sugar and 0.05 M borate. It should be noted here that none of the sugars tested by Levy and Doisy (1929) showed any change in optical activity in boric acid solution. However, evidence regarding the possible combination of boric acid with those sugars (fructose, galactose, and xylose) which show high values for combined sugar in Table II might be obtained by the use of Barfoed's copper reagent or by the cryoscopic method used in this paper. Those sugars (glucose, mannose, arabinose) which give values for combined sugar approximating 0.1 M, have pH values very close to 7. Rhamnose, maltose, and lactose solutions have pH values above 8 and in each case the amount of combined sugar is less than 0.1 M, significantly so with lactose and rhamnose. It may be noted that maltose and lactose showed less interference by borate in the oxidation of the aldehyde group by alkaline iodine than the other sugars and that this was interpreted to indicate a lesser extent of compound formation (Levy and Doisy, 1928). We believe that the data of Table II show quite definitely that compounds of sugars and borate con-

taining 2 molecules of sugar per molecule of borate do not exist to any great extent in our solutions.

In no case was the freezing point of the solution observed to change with time, indicating that the amount of compound formed did not change and that the mutarotation of various sugars in borax solutions (Levy and Doisy, 1929) was merely due to the excess sugar. The two sugar derivatives, 2, 3, 4, 6-tetramethylglucose and methylglucoside, show no evidence of combination with borax by the fieezing point method, just as the optical method gives no evidence of combination (Levy and Doisy, 1929). This is additional evidence to show that complex formation in sugars occurs between the hydroxyl group of the aldehyde carbon atom and the hydroxyl group of carbon atom 2.

CONCLUSIONS.

1. Freezing point measurements in borax solutions of sugars show that compound formation occurs.
2. The compound which is formed is probably the product of the reaction of 1 molecule of sugar and 1 of $NaBO_2$.
3. When the aldehydic hydroxyl group is blocked, as in methylglucoside, or when the hydroxyl group of carbon atom 2 is blocked, as in tetramethylglucose, complex formation does not occur.

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THE PREPARATION AND PROPERTIES OF VITAMIN C CONCENTRATES FROM LEMON JUICE.*

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The preparation and properties of vitamin C concentrates have been recently studied by several investigators. Zilva and coworkers (1) have succeeded in obtaining a concentrated material from lemon juice and have made important contributions relative to the molecular weight of the vitamin and its stability toward oxidation. Scotti-Foglieni (2) prepared a concentrate and subjected it to distillation *in vacuo*. Solubility studies have been carried out by Vedder and Lawson (3) and by Hart, Steenbock, and Lepkovsky (4).

In the present investigation an active concentrate from lemon juice was subjected to esterification and distillation, but no evidence was found to indicate any distillation of the vitamin. Fractional precipitation of the active material was then carried out, lead salts, as indicated by Zilva's work, and organic solvents being used.

A vitamin C concentrate prepared according to the method of Zilva (1927) from a liter of lemon juice was dried for an hour at 50° *in vacuo*, partially dissolved in 15 cc. of absolute ethyl alcohol, then saturated with dry hydrogen chloride, and allowed to stand at room temperature for 15 hours. The esterified product was distilled *in vacuo* at 50° and then up to 250°. The lowest pressure reached in the 50° distillation was 0.2 mm. The lowest pressure reached in the 250° distillation was 0.03 mm. The distillation was continued at 50° for 90 minutes, the receiver changed, and the temperature slowly raised through a period of 150 minutes to 250° at which it was held for 30 minutes. Distillates and residues ob-

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tained at both temperatures were fed to guinea pigs, with the results shown in Table I. The procedure followed for measuring the activity of the fractions was essentially that described by Sherman and coworkers (5).

It is evident from Table I that the distillates obtained at both temperatures were inactive, and that the residue from the 250° distillation was practically inactive. The residue from the 50° distillation had practically the same activity as the original lemon juice.

TABLE I.

Vitamin C Concentrates Subjected to Distillation after Esterification (Test Period 56 Days).

Lemon juice equivalent fed daily.	No. of animals.	Average scurvy score.*	Average gain on test.	Average survival on test.
			gm.	*days*
0	2	19	−93	35
1.5 cc. lemon juice	2	0	+155	56
3 " " "	2	0	+173	56
1.5 " residue from 50° distillation	3	3	+146	56
3 " " " 50° "	3	0	+173	33
35 " distillate " 50° "	3	18	−107	33
10 " " " 250° "	3	11	−65	35
5 " " " 250° "	3	20	−80	43

* The highest possible score is 24.

Extraction of Lemon Juice with Butyl Alcohol.

The observation that butyl alcohol extracted the yellow color and wax from lemon juice led to an experiment to determine whether vitamin C was extracted by this means. During the decitration and precipitation procedure it had been found that the yellow color was parallel with the active phase. 100 cc. quantities of juice were extracted three times with 20, 10, and 10 cc. portions of butyl alcohol. The material from the butyl alcohol extracts after evaporation and the lemon juice fraction were fed to guinea pigs in quantities of 1 and 2 cc. of lemon juice equivalents. The results of the feeding experiments are given in Table II. The butyl alcohol extraction failed to remove an appreciable amount of vitamin C

from the water phase but did remove approximately 0.33 mg. per cc. of fatty material, thus proving to be a valuable step in the concentration of the active material.

A New Method of Preparing Vitamin C Concentrates from Lemon Juice.

Zilva's method (1924, 1927) of preparing vitamin C concentrates from lemon juice involves long and tedious evaporations and leaves magnesium acetate and ammonium acetate in the final product. The average total solids found in the final fraction were 1.1 mg. per cc. of lemon juice equivalent. Since the acetates are soluble in all of the solvents in which vitamin C is known to be soluble, they are hard to separate from the final product by means of solvents.

TABLE II.

Extraction of Lemon Juice with Butyl Alcohol (Test Period 48 Days).

Lemon juice equivalent fed daily.	No. of animals.	Average scurvy score.*	Average gain on test.	Average survival on test.
			gm.	*days*
1 cc. lemon juice..................	3	3	+121	48
1 " water fraction...............	4	3	+116	48
2 " " "	5	0	+143	48
1 " alcohol "	5	19	−96	31
2 " " "	5	18	−52	33
0.............................	2	16	−100	28

* The highest possible score is 24.

To avoid these difficulties the following procedure was adopted. A slightly less than theoretical amount of basic lead carbonate, 10 to 12 gm. depending upon the acidity of the lemons, was added to lemon juice and stirred slowly until the evolution of carbon dioxide ceased (2 to 3 hours). The crystalline precipitate was filtered off after cooling and the filtrate fermented with Fleischmann's yeast. The fermentation was practically complete after 24 hours, and took place a little more rapidly if a few drops of 20 per cent phosphoric acid were added and the solution centrifuged before the yeast was added. After fermentation was complete, the yeast was centrifuged out and 17 cc. of a saturated solution of neutral lead acetate were added to each 100 cc. of decitrated lemon juice. A white, inactive precipitate resulted which was removed by centrifuging. The

filtrate containing excess lead acetate was brought to a pH of 7.2 to 7.4 (by use of either phenol red as indicator or a quinhydrone electrode), with dilute ammonia and the yellow precipitate containing most of the activity removed by centrifuging. This was dissolved in dilute acetic acid, made up to one-half the original volume with water, and the precipitation with ammonia repeated. The second active precipitate was treated with an excess of dilute hydrochloric acid and after all of the yellow precipitate had been converted over to white lead chloride, the mixture was extracted three times with 10 cc. portions of butyl alcohol. The lead chloride was precipitated from the water phase by the addition of

TABLE III.

New Method for Preparation of Vitamin C Concentrates from Lemon Juice (Test Period 56 Days).

Lemon juice equivalent fed daily.	No. of animals.	Average scurvy score.*	Average gain on test.	Average survival on test.
			gm.	*days*
1 cc. lemon juice	4	3	+100	56
1 " active precipitate, deleaded with HCl and alcohol	5	5	+106	56
1 cc. after precipitation of solid with acetone	4	5	+36	56
2 " absolute acetone extract	3	3	+28	56
2 " solid not extracted by absolute acetone	5	19	−107	27
0	5	19	−112	29

* The highest possible score is 24.

sufficient ethyl alcohol to bring the concentration up to 90 per cent alcohol. After the removal of the lead chloride, the filtrate was evaporated to dryness and then made up to one-fifth the volume of lemon juice represented with water containing a trace of acetic acid. These preparations were made at least once a week and were stored in an ice box in small flasks filled with carbon dioxide.

The results of feeding this preparation to guinea pigs are given in Table III. It will be observed that the procedure involved very little loss in the antiscorbutic value of the preparation.

Since a large amount of the total solids in the preparation as obtained by the above method was ammonium chloride, it was

thought that this could be largely removed by the addition of acetone to a concentrated water solution of the preparation. The results in Table III show that most of the vitamin remained in solution in the acetone phase. The total solids after this step were reduced to 0.5 to 1 mg. per cc. of lemon juice equivalent.

Although it has been reported that vitamin C is insoluble in absolute acetone, it was thought that it might be soluble from a more concentrated material than desiccated lemon juice. The material from the acetone precipitation was carefully dried *in vacuo* and the dried material extracted with 50 cc. of absolute acetone (dried over sodium amalgam) for 24 hours. The results are given in Table III. It might be mentioned that this experiment was carried out with lemons which were very low in antiscorbutic value. This unfortunate occurrence somewhat obscured the results of this experiment, but the vitamin was definitely soluble in absolute acetone, as the guinea pigs that survived were gaining rapidly at the end of the test, when better lemons had been obtained.

Analysis of the absolute acetone extract gave the following results:

Total solids....................	0 38–0 6 mg. per cc. lemon juice.
" N	3 46 per cent of total solids.
Ammonia N	1 9 " " " " "
Reducing sugars as dextrose...19 0	" " " " "

The final active material gave a faint carbylamine test for amino nitrogen, reduced potassium permanganate and ammoniacal silver nitrate quickly, and gave a faint coloration with ferric chloride. Bromine water produced a slight cloudiness. The phthalic anhydride and Liebermann reactions for phenols were too faint to be considered positive.

Further extraction of the dried acetone-soluble material with absolute ethyl ether, in which the vitamin is practically insoluble, reduced the total active solids to 0.28 mg. per cc. of lemon juice.

SUMMARY.

A new procedure for concentrating vitamin C from lemon juice is described.

Treatment of the vitamin C concentrate with ethyl alcohol and anhydrous hydrogen chloride, followed by distillation *in vacuo*, gave no evidence of esterification or distillation of the antiscorbutic factor. There was very little loss in activity of the preparation after it stood 15 hours in a saturated alcoholic solution of hydrogen chloride at 25–50°.

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OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL SIGNIFICANCE.

VI. THE MECHANISM OF THE CATALYTIC EFFECT OF IRON ON THE OXIDATION OF CYSTEINE.

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INTRODUCTION.

This paper is an attempt to utilize the facts presented in preceding communications of this series (1, 2) in establishing a theory of the catalytic effect of iron salts upon the oxidation of sulfhydril compounds such as cysteine.

The following facts led to the establishment of the theory:

1. Cobaltous salts combine with cysteine to form a complex which can easily be oxidized to another stable, brown complex, in different ways: (*a*) By means of ferricyanide. In this case, potentiometric titration shows two distinct successive steps of oxidation, each consuming 1 equivalent of a monovalent oxidant for 1 atom of cobalt combined in the complex. (*b*) By means of indophenol. In this case, potentiometric titration shows a smooth curve with no steps, yet the amount of oxidant consumed to reach the end-point of titration is equivalent to the total amount of ferricyanide consumed. (*c*) By means of molecular oxygen. The total amount of oxygen consumed is equivalent, also, to the amount of oxidant in the potentiometric titration with other oxidants.

2. This oxidized, brown, cobalt-cysteine complex contains cobalt atoms and cysteine molecules in the ratio 1:3. The affinity of this complex formation is strong, the reaction between cobalt and cysteine is complete, and no other complex, in the oxidized state, besides the one mentioned in the ratio 1:3 can be detected, at least in a pH range of 7 to 9.

3. The amount of oxidant consumed by a cobaltous salt and cysteine to produce the brown oxidized complex may, therefore, be said also to be two-thirds of what would be necessary to oxidize the cysteine of the complex to cystine. But no free cystine is formed—the brown complex is a rather stable end-product of the oxidation.

The fundamental idea of the following theory is the hypothesis that the oxidation of ferrocysteine leads to a product analogous to the oxidized cobalt complex, the only difference being that the oxidized iron complex is labile and forms but an intermediary stage of the iron catalysis, which finally leads to cystine. The intermediary compound of the iron catalysis, is, so to speak, stabilized when cobalt is used in place of iron.

Another fact will be given in the last section of this paper; *i.e.*, that in the oxidation of cobaltous cysteine by free oxygen no hydrogen peroxide can be detected, whereas in reactions apparently quite analogous to it, *e.g.* the oxidation of cobaltocyanide by free oxygen, hydrogen peroxide does arise.

These are the facts and the fundamental hypothesis on which the theory of the iron catalysis is based. The most hypothetical part of the theory is the fact that an analogy from certain cobalt compounds with certain iron compounds is made which cannot be strictly proved by chemical analysis. The proof of this analogy may be demonstrated by further experimental tests; but even now, this analogy is not based on a vague assumption but on recognized principles in the chemistry of metals of the iron group.

General Principles in Chemistry of the Metals of the Iron Group.

Fe, Co, and Ni are either bivalent or trivalent. For Ni the trivalent state in solutions of its normal or complex salts is so unusual and, if obtainable at all, so labile that it is quite unbelievable that the nickelic state would be ever established by exposing any nickelous compound to the air.

For Co, in many compounds, the cobaltous state is the only stable one. In other compounds the tendency of the cobaltous state to oxidation is so high that, in the presence of air, the cobaltic state is the only stable one. Seemingly there are scarcely any cobalt compounds, which by moderately strong oxidants and reductants can easily be shifted from the cobaltous state to the cobaltic and *vice versa*, as is the case with iron compounds. Those

cobalt compounds which are stable only in the cobaltic form are complex compounds which contain quite definite molecules or ions, among which ammonia, cyanide, and nitrite may be mentioned. For the sake of brevity, such atom groups which call out the third valence of cobalt will be referred to as *valence exciters*. The first thesis to be used in the arguments to follow is this:

1. Cysteine (or thioglycolic acid and other sulfhydril bodies containing a carboxyl group) is a valence exciter for cobalt. This thesis is nothing but the expression of the fact that cobaltocysteine is rapidly oxidized by air. At least a part of this oxidation must concern the change of valence of the cobalt atom.

2. The number of coordination places for complex formation, according to Werner, is six both for Fe and Co. When an anion is attached to the metal ion by valence, one positive charge of the metal ion is neutralized by the negative charge of the anion. When an ion is attached by a residual valence, its electric charge is maintained. The latter statement may be also expressed by saying: when an acid is fixed by a residual valence, its H atom is maintained.

3. Sulfhydril bodies have a strong tendency to form a disulfide, as soon as the H atoms are detached from the SH group.

4. All reactions of cysteine with these metals take place with thioglycolic acid also. The amino group is not involved in this reaction. Thioacetic acid reacts differently. Cysteine ethylated at the SH group gives no color reactions at all. The reaction of cysteine, in which we are interested here, is therefore due to the simultaneous presence of an SH and a COOH group in the molecule.

It cannot be contended that the amino group of cysteine has no affinity for cobalt. It may be that it does not react with cobalt only because 1 molecule of cysteine cannot occupy more than two places of coordination, of which one is taken by the SH group, the other by the COOH group. So we suppose in the fourth place that cysteine can occupy no more than two valences or coordination places.

Application of These Principles to Complex Compounds of Cysteine with Cobalt.

Let us now infer from these suppositions what reactions may be expected when cysteine and a salt of these metals act upon each other.

The complex of cysteine with nickel is not capable of oxidation. It is of no interest in this discussion, except for its probable analogy with the cobaltous or ferrous complexes. This analogy, however, may not be complete because nickel complexes usually have only four coordination places occupied.

Cobalt may be able to give simple or normal salts with cysteine. However, the conditions of our experiments always require a slight alkalinity, pH 7 to 8.5, and at this pH, in the absence of cysteine, cobalt is present as a precipitate, according to the buffer used as a basic phosphate or borate. When cysteine is added, it dissolves this precipitate, and immediately produces a higher complex instead of a normal salt. The ratio of cysteine molecules to cobalt atoms is 3:1 in the oxidized form of the complex, when it is formed at a pH 7 to 9. This holds for the oxidized complex. It is therefore very probable that the cobaltous complex, or better, that particular cobaltous complex from which the oxidized complex originates, has the same ratio. There may be different kinds of cobaltous complexes with cysteine as suggested by the different colors of the cobaltous complex according to the ratios used in mixing cobalt and cysteine (Michaelis and Barron (1)) but that particular complex which is considered as a stage preliminary to the oxidized complex, will have the same ratio, namely 3:1. This assumption may be, at least roughly, confirmed by an observation made in performing the potentiometric experiments (1), that in the absence of air about 3 molecules, at least more than 2 and certainly not more than 3 molecules, of cysteine are necessary to dissolve 1 atom of cobalt, which is present as a precipitate of basic cobalt phosphate. Such a complex will have the constitution $Co^{II}(SR)_3H_4$, when cysteine is symbolized by HSRH, where R stands for $CH_2 \cdot CHNH_2 \cdot COO$.

This cobaltous complex will have the form shown in Formula I (Fig. 1). The two main valences must be thought of as attached to sulfur, which has a greater affinity for Co than has a carboxyl group.[1] As the third sulfhydril group is a valence

[1] It may be briefly mentioned here that from the standpoint of Werner's octahedral arrangement of the six coordination places a complex of this structure is possible in four isomeric forms; there may be two isomers with respect to structure, either of them in two mirror-symmetric forms. Perhaps even a greater variety is possible owing to the asymmetry of the cysteine itself.

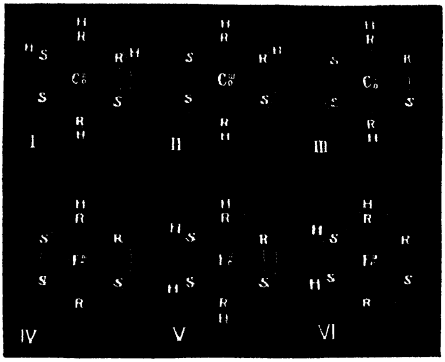

FIG. 1. R represents CH_2 $CHNH_2COO$. Cysteine is, therefore, HSRH; cystine is HRSSRH. Main valences are represented by solid lines; residual valences by dash lines.

Formula I. Cobaltous tricysteine. The two valences are attached to S groups, not to R groups. The asymmetry with respect to the spacial distribution of the two valences is only apparent and disappears when the plane figure is substituted by the octahedral distribution of the six coordination places around the central metal atom, according to Werner. Instead, other possibilities of isomerism arise (see foot-note 1). Ferrous tricysteine must be considered analogous to this complex.

Formula II. Cobaltic tricysteine. This arises from Formula I by loss of 1 H atom. The third valence is attached again to S, not to R. Ferric tricysteine is analogous.

Formula III. Cobaltous cysteine-cystine. This arises from Formula II by loss of another H atom. One valence is attached to S, the second, however, necessarily to R because no SH group is available. Therefore this complex cannot undergo a further dehydrogenation and must remain in the cobaltous state. Ferrous cysteine-cystine is analogous to this complex, but here further dehydrogenation is possible, which would produce Formula IV.

Formula IV. Ferric cysteine-cystine. When in ferrous cysteine-cystine (Formula III with Fe instead of Co) 1 molecule of cystine is replaced by 2 molecules of cysteine, there arises Formula V.

Formula V. Ferrous tricysteine. This differs from the structure represented by Formula I (with Fe instead of Co) only by the distribution of the main valences. On account of the greater affinity of iron for S than for R, Formula V will shift spontaneously to Formula I and the cycle is closed.

In case that Formula III is oxidized to Formula IV before an exchange of 1 cystine molecule for 2 of cysteine can take place, this exchange will occur in Formula IV instead of in Formula III. This leads to Formula VI, and then the rearrangement of the three main valences in such a way that they become attached to S instead of R, resulting in Formula II (with Fe instead of Co) takes place. A cycle is closed in this way also.

exciter for cobalt, this complex will in the presence of an oxidant or free oxygen lose 1 H atom (or, considered as a quadrivalent ion, it will detach one electron and become a trivalent ion) and the cobaltic complex, Formula II, will arise.

Now, 2 neighboring S atoms freed from hydrogen, will have the tendency to form a disulfide. After the 2 S atoms have been linked to each other by a true valence, they no longer can be attached to the central cobalt atom by main valences, but only by residual valences, and now we must dispose of the two lost main valences. One will be restored in such a way that one of the RH groups is now bound by a main valence. This involves the loss of the H atom belonging to this RH group (Formula III) and finds its experimental equivalent in the second step of oxidation. The other lost main valence will not be restored but is definitely lost. As there is no valence-provoking SH group left, cobalt will stay in the bivalent state. The second step of oxidation, remarkably enough, involves the return of the cobaltic state to the cobaltous. The definite balance of oxidation is now that the original cobaltotricysteine complex, Formula I, has lost 2 hydrogen atoms, that the cobalt atom has returned to its bivalent form, and that two sulfhydril groups are combined to form a disulfide group. Thus we find that, starting from the original complex, Formula I, 2 hydrogen atoms have been detached for each atom of cobalt or for each 3 molecules of cysteine, which corresponds to the amount of oxygen consumption found in the experiments.

This cobaltocysteine-cystine, therefore, is the end-product of the reaction, provided that the reaction takes place under such conditions, especially concerning pH, as were used in the experiments of the recent papers.

Necessity of Two Step Oxidation.

It may seem to be an easier way of obtaining the oxidized complex, Formula III, from Formula I, if 2 hydrogen atoms of Formula I are removed by the action of an oxidant at the same time. It is justifiable to raise the question why this simpler formulation of the oxidation is discarded. This question may be answered in the following manner. The oxidation in two steps, from Formulas I to II to III, involves the intermediary transition of the cobalt from the bivalent state to the trivalent, whereas no change of the

cobaltous state would be involved in the direct two step oxidation of Formula I to III. If a change of valency were not necessary, the oxidation should be expected to go on just as easily with a complex of a non-oxidizable central metal atom. This, however, is not the case. Oxidation takes place with Fe,Cu, Mn, but not with Ni though Ni also gives colored complexes with cysteine. The number of coordination places in nickel is according to analogous nickel complexes, usually at most four instead of six as in the case of cobalt. These four places may be occupied by 2 molecules of cysteine. This difference however, is not a sufficient reason to reject the possibility of oxidation. For with copper also the maximum number of coordination places is usually four; *e.g.*, $Cu(CN)_4K_3$ in contrast to $Co(CN)_6K_3$ or $Fe(CN)_6K_3$. Yet the copper complex of cysteine is easily oxidized. The nickel complex of cysteine, however, cannot be oxidized by oxygen or any dyestuff, corresponding to the general reluctance of nickel to enter the nickelic state. Obviously the breaking up of this oxidation into two steps due to a cyclic change of the valence of the metal is the essential feature of this process. In order to oxidize 2 molecules of cysteine to 1 molecule of cystine, 2 hydrogen atoms must be taken up by an acceptor. This seems to be difficult, at low temperature, with oxidants of a mild character as dyestuffs, or of a sluggish character as molecular oxygen. The oxidation is facilitated by dividing this oxidation into two successive steps, each involving 1 hydrogen atom. This is achieved by the intermediation of a metal of easily changeable valence. Hence, the transition of Formula I into III goes on only via Formula II.

Iron Complexes.

We now state the hypothesis that the reaction with iron is the same as with cobalt up to the formation of the oxidized cobalt complex, but that the ferrocysteine-cystine complex is less stable than the corresponding cobalt complex.

There are two possibilities that the ferrocysteine-cystine complex may undergo further alterations. One is based on the assumption that the stability of the iron complex is less than that of the cobalt complex. The experimental justification for this assumption is the fact, described by Michaelis and Yamaguchi (2), that cysteine combined with cobalt in the oxidized complex

is not catalytically oxidized to cystine by oxygen gas in the presence of iron salts. The affinity of cysteine for cobalt is so great that iron salts are not able to withdraw cysteine from the cobalt complex. In other words, the affinity for the formation of the iron complex of cysteine must be much less than for the formation of the cobalt complex.

The other possibility is based on the assumption that a ferrous compound analogous to Formula III can be oxidized to a ferric compound even without a specific valence exciter. According to whether we consider the first or second possibility, there are two routes along which the catalysis may proceed.

Let us follow the first route. As the complex with iron is looser than the complex with cobalt, it may partially dissociate into its constituents, one of which is cystine; furthermore there may be an exchange of the cystine combined with this complex, for 2 molecules of cysteine which are free in the solution, and the main valences may be rearranged so as to agree with Formula I. This exchange may be primarily a reversible reaction tending towards an equilibrium, but it is made irreversible by the fact that the ferrotricysteine arising from this exchange, will necessarily start over again the whole process of oxidation. Thus all cysteine is gradually transformed to cystine, even with a very small amount of iron present.

Let us now follow the second route. Ferrocysteine-cystine (Formula III with Fe instead of Co) may be oxidized by a further step of oxidation to ferricysteine-cystine (Formula IV), and the same exchange of 1 molecule of cystine for 2 molecules of cysteine takes place as just described. Now, as the disulfide group is replaced by two SH groups, the tendency of the main valences for combining with SH rather than with COOH can be satisfied, and what results is ferritricysteine. This is, again, one of the complexes encountered before (Formula II), and a cyclic process is likewise started.

It should be left to a further investigation to determine whether these two routes of the catalysis take place simultaneously or whether a preference is given to one of them.

The Problem of H_2O_2 Formation.

When free oxygen works as a dehydrogenator, the reduction of the oxygen will go primarily to H_2O_2. This has been proved

experimentally in many cases; *e.g.*, when reduced indigosulfonate is oxidized by air, H_2O_2 arises. In contrast to this, when cysteine is oxidized by free oxygen with a trace of iron as catalyst, no H_2O_2 can be detected. The same holds, as we may add now, for the oxidation of cobaltocysteine to the brown complex by molecular oxygen. If this fact could be brought into agreement with the present theory, it would be further support for it. The lack of H_2O_2 formation in this case is all the more striking as there is another instance, very well known, in which a cobalto complex is oxidized by free oxygen, and H_2O_2 *is* detectable, not only qualitatively, but almost quantitatively up to the theoretical amount. This is the cyanide complex of cobalt. As cysteine and cyanide behave rather similarly with respect to the affinity for heavy metals and the inclination to form complexes, a comparative study of the cyanide—and the cysteine—complexes will be important. Our theory must account for this difference in the H_2O_2 formation if it is to be acceptable.

Cobaltocyanide is exceedingly less stable than ferrocyanide. Cobaltocyanide is, in the presence of air, spontaneously and very quickly oxidized to cobalticyanide, cyanide being a valence exciter for cobalt. In the absence of air, cobaltocyanide even develops hydrogen gas from the water, especially in the presence of platinized platinum (Zwenger (3), Manchot and Herzog (4)). When it is oxidized by air, twice the expected amount of oxygen is absorbed, and besides cobalticyanide an almost equivalent amount of H_2O_2 is formed and remains in the solution. Only on boiling does the H_2O_2 deliver oxygen. On the other hand, on oxidation of cobaltocysteine by aii, no H_2O_2 can be detected. The difference is due evidently to the following circumstance. The oxidation of cobaltocyanide consists in the detachment of 1 H atom (or loss of one electron). Cobaltocyanide is simply oxidized to cobalticyanide. Each pair of the H atoms detached by 2 molecules of cobaltocyanide combines with 1 molecule of O_2 to form H_2O_2.

Such an experiment can be performed in a test-tube in this manner. 1 cc. of 0.1 M cobalt sulfate or nitrate is mixed with enough KCN to dissolve the initial precipitate and exposed for a short time to the air until the greenish color has turned slightly yellow. Now an equal volume of ether is added, several drops of

a 5 per cent solution of chromic acid are added, and the mixture shaken. When chromic acid is added in such amount as to make the solution just slightly acid, the ether turns blue; this is a test for H_2O_2 (excess of chromic acid destroys the color again). When the analogous experiment is made with cysteine instead of cyanide, the test for H_2O_2 is negative.

Thus, cobaltocyanide appears analogous to cobaltocysteine in one respect,—*i.e.* ease of oxidation, but different on the other hand,—namely with respect to the amount of oxygen consumed and the formation of H_2O_2. In cobaltocysteine, the oxidation takes place in two successive steps, as Michaelis and Barron have shown by a potentiometric method, upon oxidation with a single step oxidant such as ferricyanide. Only the first step can be analogous to the oxidation of cobaltocyanide.

The two steps manifested in potentiometric titration must exist also in the oxidation with oxygen gas. Gas analytical methods, of course, do not detect it, but the lack of formation of H_2O_2 shows it. Cobaltocysteine primarily reduces O_2 to H_2O_2, and before this H_2O_2 can escape from molecular contact with the cobalt complex, it is reduced in the second step of the process to H_2O. Either partial process of the oxidation of the cobalt complex is a dehydrogenation, when we consider the formulas as given in Fig. 1. We may replace these structures just as well, or even in a more adequate way, by showing the hydrogen atoms of the outside sphere of the complex detached in the form of free hydrogen ions, leaving electrons attached to the complex. In this case either step of oxidation involves the loss of an electron.

It is important to emphasize that the interpretation of this oxidation as dehydrogenation does not exclude the possibility that an oxygen addition compound is formed as a stage preliminary to the dehydrogenation if molecular oxygen is used as an oxidant. This is the more probable as the carbon monoxide compounds of ferrous and of cobaltous cysteine described by Cremer (5) suggest the existence of similar oxygen compounds. These oxygen addition products, if there be any, can, however, only be considered as a transient stage. The discussion of the existence of these intermediary oxygen compounds should be left to a special investigation.

SUMMARY.

Cobaltous salts, at pH 7 to 8, form complexes with cysteine, among which one is particularly prevalent. It may be designated as cobaltotricysteine. This is oxidized in two steps, the first leading to cobaltitricysteine, the second step leading to cobalto-cysteine-cystine. The latter is stable. Ferrous salts give the same reactions, but here, the ferrocysteine-cystine complex reacts with 2 molecules of free cysteine so as to furnish free cystine and ferrotricysteine. This involves a cyclic process which ends in the oxidation of all cysteine to cystine. When oxygen gas is the oxidant, it is completely reduced to H_2O. The first step of the oxidation probably reduces the oxygen to H_2O_2, the second to H_2O.

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